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HybriGene LLC

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October 19, 2004

Regulatory Analysis and Development,
PDD, APHIS, Station 3C71
4700 River Road Unit 118
Riverdale, MD 20737-1238

RE: Docket No. 03-101-2

In response to the call for comments regarding the Environmental Impact Statement to be prepared by APHIS and consideration of a petition submitted by Monsanto and the Scott's Company, this reply is intended to summarize the problem.

Whereas the Roundup gene has now escaped from the control area as indicated it would by the Pollen Flow Study done by Dr. Joseph Wipff and Crystal Fricker of Pure Seed Testing in 1998-1999[1] and as stated in testimony at hearings held by the Oregon Department of Agriculture and at the CAST Workshop held in Baltimore, MD on January 9th and 10th, 2003;

Whereas the Jefferson County irrigation district is now contaminated with this gene, as evidenced by the Environmental Protection Agency study "Evidence for landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with CP4 EPSPS as a marker" just published in PNAS, October, 2004[2];

Whereas the study showed the gene was found 13 miles from the control area;

Whereas a study performed by Tee-2-Green Corp with sentinel and residential plants outside of the control area shows many progeny *Agrostis* plants resistant to the herbicide;

Whereas HybriGene and Pure Seed Testing have a pollen control system that has demonstrated no escapes of viable transgenic glufosinate resistant pollen in tests conducted in 2002 and 2003, and with the 2004 tests now being sprayed and examined by microsposy. The 2002 and 2003 tests were all conducted in a secure greenhouse. The 2004 study was intended to be an outside test as acknowledged according to Notification no. 04-086-04n, but moved inside the secure greenhouse June 16th, as mandated by Virgil Meyer and Neal Hoffman;

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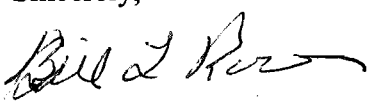
Therefore it seems most urgent that no further dispersement of transgenic pollen containing the Roundup gene be allowed from the present control area and an intensive effort be made to kill bentgrass plants that are resistant to Roundup as indicated by the Trait Test wherever they are found in this entire irrigation district. It is also most urgent to continue the Environmental Protection Agency study being conducted in Corvallis, Oregon to determine the longevity of these contaminated resident and sentinel plants to see if they survive or dominate in the environment.

Sincerely,



Bill L. Rose

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[1] Wipff, J.K., and C. Fricker. 2001. Gene flow from transgenic creeping bentgrass (*Agrostis stolonifera* L.) in the Willamette Valley, Oregon. International Turfgrass Society Research Journal 9:224-242.

[2] Watrud, L.S., E.H. Lee, A, Fairbrother, C. Burdick, J.R. Reichman, M. Bollman, M. Storm, G. King, and P.K. Van de Water. 2004. Evidence for landscpe-level, pollen mediated gene flow from genetically modified creeping bentgrass with CP4 EPSPS as a marker. Proceedings of The National Academy of Sciences 40:14533-14538.

International Turfgrass Society
Research Journal Volume 9, 2001.

GENE FLOW FROM TRANSGENIC CREEPING BENTGRASS (*AGROSTIS STOLONIFERA* L.) IN THE WILLAMETTE VALLEY, OREGON

J. K. Wipff and C. Fricker

ABSTRACT

Since the Willamette Valley produces nearly all of the bentgrass (*Agrostis* spp.) seed grown in the United States and exports bentgrass seed in large quantities to Europe, risks associated with the commercial production of transgenic cultivars in the major grass seed production area of the world must be defined. Bentgrass can be a serious weed in other crops. Since creeping bentgrass may be the first perennial, wind pollinated, outcrossing, transgenic grass species to be produced commercially, essential data must be generated on pollen movement within the crop. Pollen flow, viability, pollination and seed formation are all sensitive to climatic parameters, which differ greatly from region to region, so it is imperative that this research be conducted where the crop will be commercially cultivated. The two primary objectives of this study were to investigate intra- and interspecific gene flow of transgenic creeping bentgrass in the Willamette Valley of Oregon. Pollen movement was determined by placing transects of non-transgenic creeping bentgrass around a nursery of 286 transgenic plants genetically engineered for tolerance to the herbicide glufosinate. Approximately 250 non-transgenic creeping bentgrass plants were planted in transects around the transgenic nursery in 1998 and 1999 near Hubbard, OR. In 1998, the following transects were established: 1) two circles around the nursery at 109 (33.2 m) and 272.5 ft (83.1 m) with plants spaced at 50 ft (15.24 m) and 100 ft (30.48 m), respectively; and 2) two line transects aligned with prevailing winds (NE) with one transect NE 244 ft (74.4 m) and the SW transect 370 ft (112.8 m) from the edge of the nursery based on Oregon Seed Certification isolation distances, which is 165-300 ft (50.3-91.4 m) (depending upon field size) for certified seed production. In 1999, the length of the line transects were increased to the following: 1) SW transect, 978 ft (298.1 m); 2) NE transect, 268 ft (81.7 m); 3) SE transect, 612 ft (186.5 m); and 5) NW transect, 319 ft (97.2 m). Using non-linear regression, the following distances were predicted for transgenic pollen introgression to the 0.02% level. In 1998, along the SW transect, transgenic pollen traveled 3,500 ft (1,066.8 m) and along the NE transect it traveled 4,296 ft (1,309.4 m). In 1999, the transgenic pollen was estimated to have traveled 1,022 ft (331.5 m) to the SW, 1,892 ft (576.7 m) to the NE, 861 ft (262.4 m) to the NW, and 1,022 ft (331.5 m) to the SE. The second part of the study was to evaluate interspecific hybridization in creeping bentgrass for its propensity to hybridize freely. Twelve species of bentgrass have been documented as occurring in the area of bentgrass seed production. Six are naturalized species and are part of a complex that freely hybridize: *A. canina* L., *A. capillaris* L., *A. castellana* Boiss. & Reuter, *A. gigantea* Roth, *A. stolonifera*, and *A. vinealis* Schreber. Accessions of *A. canina*, *A. capillaris*, *A. castellana*, *A. curtissii*, *A. gigantea*, *A. pallens*, and *A.* sp. were placed in the transgenic nursery prior to flowering and allowed to interpollinate. The crossing experiments resulted in the introgression of the bar gene from creeping bentgrass into *A. canina*, *A. capillaris*, *A. castellana*, *A. gigantea*, *A. pallens*, and *A.* sp. These data will be available to USDA-APHIS for use in creating a risk assessment model for commercial transgenic grass seed production.

INTRODUCTION

The importance and value of turfgrass is accelerating, due to its association with an ever-increasing urban population. In the United States alone there are over 14,000 golf courses, 40,000 athletic facilities, and 40 million home lawns and parks [Edminster, 2000]. This importance can also be seen in the size of the turfgrass seed market, which is only second to that of hybrid seed corn [Lee, 1996], with annual sales between $580 million – 1.2 billion. Although turfgrass management and production is one of the fastest growing areas of agriculture, genetic transformation of turfgrasses lags behind that of many other important crop plants [Johnson and Riordan, 1999]. Though conventional plant breeding can incorporate individual and multiple stress tolerance, as well as, agronomic traits, there are biological limits to how far a species can be manipulated by traditional plant breeding methods and progress can be extremely slow, costly, and sometimes impossible. DNA biotechnology can provide new, efficient, and innovative methods for the incorporation of not only individual, but also complex, multiple abiotic and biotic stress tolerances [Dale, 1993; Lee, 1996; Brilman, 1997; Duncan and Carrow, 1999] that would be extremely difficult, if not impossible using classical plant breeding methods.

DNA biotechnology expands the available gene pool to include every living thing. Primary, secondary, or tertiary gene pools [as defined in Harlan and de Wet, 1971] in traditional plant breeding no longer exist, but anything with DNA is now in the primary gene pool.

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Transformation technologies allow plant breeders to combine the genes for a trait from different species. In conventional plant breeding, the level of difficulty in developing new cultivars is related not only to the genetics and complexity of the trait(s) of interest, but also to the number of undesirable traits or characteristics in the parents, which is dependent on which gene pool the parent belongs. When a trait(s) is transferred via traditional plant breeding methods it carries a number of undesirable traits which must be removed. The further down the gene pool that a desired trait is located, the more undesirable genes that must also be dealt with. With DNA biotechnology, only the gene(s) of interest are incorporated into the plant genome; thus no time is needed for repeated backcrosses to remove undesirable genes.

DNA biotechnology offers two new types of tools for plant breeders: Genomics and Plant Transformation [Sage, 1999]. Genomics involves the technologies of mapping and selecting genes. This provides a tool for the plant breeder to track the genes controlling comparable traits in different species. Plant transformation involves building genes into constructs that can then be rapidly moved across species. Genomics can be divided into two further divisions: Structural and Functional. Structural Genomics is the process of determining gene location in a genome and has led to discovery of colinearity or synteny: the conservation of gene order on chromosomes across different species within families. Functional Genomics is the process of finding out what individual genes control at the biochemical level, which leads to the discovery of many new and potentially economically important traits. Microarray analysis provides a way to link genomic sequence information (Structural Genomics) with Functional Genomics [Lemieux et al., 1998]. Future techniques will be based on DNA sequences in the genes themselves, rather than by linked markers [Barker and Warnke, 1998]. Sequence recognition will be done using DNA microarrays and chip technology. DNA chip technology utilizes microscopic array (microarrays) of molecules immobilized on solid surfaces for biochemical analysis. Microarrays can be used for expression analysis, polymorphism detections, DNA resequencing, and genotyping on a genomic scale. Microarray-based characterizations of plant genomes has the potential to revolutionize plant breeding and agricultural biotechnology. Microarrys allow for the rapid analysis of transgenic plants. These data will permit genome-wide correlations between expression patterns and a host of desirable traits such as fertility, seed set, yield, and resistance to abiotic/edaphic and biotic stresses. Costly field trials could be reduced using chip-based analysis of transgenic lines. Micoarrays in gene sequencing and diagnostics will greatly escalate the identification of DNA polymorphisms, which in turn could be used to expedite plant breeding by allowing simultaneous analysis of thousands of polymorphisms in a single experiment. Thus this technology has the potential to be a powerful selection tool that could eventually replace marker assisted selection. DNA technologies will also be important in the protection of intellectual property rights by serving as the molecular identification tools to distinguish between turfgrass cultivars or to actually insert a patented DNA sequence into a cultivar as an identification tag. Plant breeding will be revolutionized as gene technologies become more efficient and economical.

DNA biotechnological enhancement is being considered for virtually all commercially important plants and the number of field tests for transgenic plants is increasing exponentially. For example in perennial grasses, the following is the number of permits/notifications have been issued or pending (number submitted) by USDA/APHIS (http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm):

Species	1993-1997	1998	1999	2000
Creeping bentgrass	11 (12)	16 (19)	25 (26)	20 (24)
Kentucky bluegrass	—	1 (2)	8 (8)	7 (7)
Perennial ryegrass	—	—	1 (1)	1 (1)
Tall fescue	—	—	2 (3)	1 (1)
Bermudagrass	—	—	2 (2)	2 (2)

Many of the applications of DNA engineering in agriculture will probably have neutral or even beneficial environmental consequences, but commercial-scale production of some of these transgenic plants could lead to undesirable environmental and agricultural consequences [Snow and Morán Palma, 1997]. Environmental consequences for the commercialization of transgenic crops [e.g. Colwell et al., 1985; Rogers and Parkes, 1995; Tiedje et al., 1989] has been debated extensively. On one side, the argument is that the phenotypes of transgenic plants are similar to phenotypes of plants developed through conventional breeding methods and that these plants are therefore not inherently risky [e.g. Brill, 1985; Miller, 1994]. On the other side, the argument is that the access to unlimited numbers of genes from unrelated organisms makes genetic engineering a new and potentially dangerous technique, with the major concern being evolution of undesirable weeds or pesticide resistant insects [e.g. Ellstrand and Hoffman, 1990; Rissler and Mellon, 1993; Tiedje et al., 1989]. These risks and concerns also apply to traditionally bred crops as well, but the release of transgenic plants has focused attention on this new technology and its potential consequences [Snow and Morán Palma, 1997].

The essence of the issue with the ecological impact of bioengineered plants is the following: 1) it is not the techniques themselves that may lead to environmental or agricultural problems, but rather the phenotypic traits that results; 2) the need for concern varies greatly depending on the type of trait(s) transferred and whether the transgenic organism can persist in free-living populations or hybridize with non-transgenic populations of the same or related species; 3) the understanding of the long term effects of the commercialization requires an interdisciplinary approach; and 4) most of the thousands

of small-scale field tests of transgenic plants have not been designed to investigate the ecological risks associated with widespread commercialization [Wrubel et al., 1992]. The dangers of increased weediness depend less on the type of gene transfer method involved in crop improvement than on the biology of the gene transferred and how it is expressed in the hybrid [Tiedje et al., 1989]. The significance of gene escape will vary with the biology of the plant donor, the wild recipient, and the introduced gene. In regards to the crop donor, primary risks will probably come from crops: 1) that have undergone little domestication because there would be minimal ecological and reproductive divergence from the wild progenitors [Ellstrand and Hoffman, 1990]; 2) grown sympatric with wild relatives (e.g. centers of origin) or cross-compatible species (or genera); 3) that have biotypes or related taxa that are already aggressive weeds; 4) that can also be weeds themselves; and 5) outcross with some degree of self-incompatibility.

Plants such as turf, forages, rangeland, and bioremediation grasses that have undergone little domestication and often have wild relatives growing in sympatry [Arriola and Ellstrand, 1996; Giddings et al., 1997a; Ellstrand and Hoffman, 1990] and/or can be weeds outside of cultivation and in other crops, are particularly at risk of spreading transgenic DNA. Bermuda grass [*Cynodon dactylon* (L.) C. Persoon] is an important perennial forage and turfgrass, but it is also, in many areas of the United States, is one of the worst weeds. If a gene that would confer a selective advantage were introduced into a cultivar (by either biotechnology or conventional breeding) and the gene escaped into the weedy populations, the immediate economic and ecological impact could be significant [Ellstrand and Hoffman, 1990]. Giddings et al. [1997a] reported that the major wind-pollinated crops in the United Kingdom that are cross-compatible with native and feral populations and that are likely to flower on a wide scale are the forage grasses. Forage rygrasses (*Lolium perenne* L. and *L. perenne* var. *aristata* C. von Willdenow (syn = *L. multiflorum* J. de Lamarck)are widely cultivated cross pollinating forage crops, which readily outcross with wild and feral populations and occasionally with fescue species (*Festuca* spp.). *Lolium-Festuca* hybrids (x *Festulolium* Ascherson & Graebner) can be fertile and have an ability to backcross with either of the parents.

These problems are not just theoretical, because within highly domesticated crops, crop-weed hybridizations have occurred, leading to the evolution of aggressive weeds [Barrett, 1983], and demonstrating that the hazards created by these hybridizations occur in nature and are not unique to genetic engineering. These weeds are difficult to control because they share so many traits with the crop. Examples of this can be found in cultivated sorghum (*Sorghum bicolor* (L.) C. Moench), pearl millet (*Pennisetum glaucum* L.), radish (*Raphanus sativus* L.), and sugar beets (*Beta vulagaris* L. subsp. *vulgaris*). Johnsongrass (*Sorghum halepense* (L.) C. Persoon) is known to be an interspecific hybrid descendant of *S. bicolor* and *S. propinquum* (K. Kunth) A. Hitchcock [Paterson et al., 1995] and is considered to be a primary noxious weed worldwide [Holm et al. 1977]. Its hybridization with cultivated sorghum resulted in the evolution of invasive weedy biotypes [Baker, 1972; Arriola and Ellstand, 1996]. A similar case happened with pearl millet. A common noxious weed (*P. sieberanum* (Schlecht.) Stapf & Hubbard) of pearl millet in Africa is also a biotype of pearl millet. This weedy biotype, which mimics pearl millet, evolved through the hybridization of pearl millet with its wild progenitor (*P. violaceum* (Lam.) Rich.) [Brunken et al., 1977]. In California during the nineteenth century, cultivated radish hybridized with an introduced weed, *Raphanus raphanistrum* L.) to create a new weed known as wild radish [Panetsos and Baker, 1967]. More recently, a new weed evolved in France when sugar beets (*Beta vulgaris* L. subsp. *vulgaris*) hybridized with a subspecies from the Mediterranean [*B. vulgaris* ssp. *maritima* (L.) Archang.] [Boudry et al., 1993]. These examples also demonstrate that new weeds can evolve quickly.

The concern regarding the transfer of alien genes into local populations that results in non-weedy populations becoming weedy or weedy populations becoming more weedy is not the only issue. The other ecological impact could be the contamination of the native gene pool, which could have a variety of possible consequences [Kareiva et al., 1994]. For example, the extinction of wild rice in Taiwan has been attributed in part to gene flow from cultivated rice to wild rice [Kiang et al., 1979; Oka, 1992]. So, the full impact of gene escapement on natural populations must be considered. If the genes and transgenic plants could be confined with certainty to the agricultural settings for which they were designed, their negative ecological and agricultural impacts would be greatly reduced. Unfortunately, pollen and seeds are dispersed, and the door is open to a cascade of environmental problems, such as, an increase in weediness and invasiveness that could threaten extinction of native plants, contamination of gene pools, and the disruption of innate plant communities [Ellstrand, 1988]. Since many of these problems begin with gene escape through the pollen, the logical first step in a biotechnology risk assessment is the quantifications of pollen movement and the intraspecific, interspecific and intergeneric hybridization possibilities between transgenic crops and non-transgenic populations. If pollen is found to travel great distances, pollen viability becomes an issue, and determining how far viable pollen travels under various environmental conditions must be assessed.

The dispersal distance of pollen from a source is dependent on a number of factors, such as wind speed and turbulence, height, and duration of pollen release [Okubo and Levin, 1989], and other climatic conditions (e.g. relative humidity, temperature, and air pressure). Giddings et al. [1997a,b] conducted a series of experiments on pollen dispersal for determining the risk of introducing genetically modified wind-pollinated forage

grasses. Pollen dispersal was studied from a perennial ryegrass (*Lolium perenne* L.) source using pollen traps. Giddings et al. [1997a] reported that the most striking feature reoccurring throughout their analysis was the high variability of pollen deposition. They found a large amount of variation in pollen dispersal over time and to traps of different orientations. Twelve data sets were collected and were used to test Bateman's equations [Bateman, 1947] for the wind dispersal of pollen. Batman's equations were found not to be useful for describing dispersal over distance and clearly needed to be modified to take factors such as wind direction into account. Giddings et al. [1997b] studying the influence of wind direction on pollen dispersal, found that for 11 of the 12 data sets, the new equation fit significantly better than Bateman's original equations. They also produced 15 data subsets from the mean wind directions to test Bateman's equations for dispersal downwind from a pollen source. These equations fit only four of the data subsets. They found that the amount of pollen deposited does not always decrease smoothly with increasing distance from the source. Three of the 12 data sets they analyzed had an increase in pollen deposition with distance; more pollen was collected at 80 m than at 60 m. This highlights the danger of assuming that deposition decreases smoothly over distance varying only with wind direction. Pollen clouds are taken high into the atmosphere, moved, and deposited during times of calm. Pollen could possibly be transported considerable distances from the source, and so could be important in terms of transgene spread. Wind speed and turbulence were expected to be important factors affecting this process. However, models involving such additional parameters would be complex and would probably show chaotic dynamics, making prediction difficult. In bentgrass production fields, one form of turbulence often observed are 'whirl winds' that move across a field and carry pollen high into the atmosphere. Since the prediction of pollen dispersal involving this kind of turbulence is extremely difficult and currently no models are known to exist that accurately predict the effects of these complex parameters, any prediction models will have to be assumed that there will be a slow decline in pollen dispersal with increasing distance from a source. Traditionally, pollen dispersal was assumed to follow a bivariate normal distribution [Wright, 1943; Haldane, 1948]. However, pollen dispersal distributions from plants are strongly leptokurtic [Levin and Kerster, 1974; Thompson et al., 1999] and this has led to the suggestion that a exponential power function [Bateman, 1947; Kareiva, 1994] be used to describe pollen distribution. Since climatic parameters will differ greatly from region to region and have an effect on pollen movement, viability, and frequency of successful fertilizations, research must be conducted where the crop will be commercially cultivated.

Giddings [2000] reported on *Lolium perenne* pollen dispersal using a Gaussian plume model, which takes into account distance and wind direction. The model was used to calculate, using integration, possible pollen deposition onto small conspecific populations one kilometer from the source. Initially the source and recipient populations were 2-m in radius (the same as the source the data is based). The percentage of immigrant pollen was compared for six different sets of parameter values previously estimated from pollen-dispersal experiments [Giddings et al., 1997a,b]. In the 2-m radius plots 0.008 to 3.52 % of the pollen deposited on the downwind "recipient" population was from the donor "transgenic" population. The source size was then scaled up to 10.24 ha (25.3 ac) to simulate what might happen if transgenic ryegrass was grown on a large scale When the source size was scaled up to 10.24 ha, the amount of pollen deposition increased to 29.74 to 99.64 % downwind from the source. Recipient populations centered 90° to the mean wind direction received 4.90 to 51.12 % of the pollen from the source population. Because of the potential for long-distance pollen movement, the use of 'guard rows' around transgenic crops to prevent gene flow out of the field seems unlikely to be effective at preventing pollen dispersal at some distance from the crop.

The study of "gene-transfer or gene-flow at the population level is a fundamental issue to risk assessment, because it represents an avenue by which engineered genes may escape from cultivated fields" [Kjellsson et al., 1997]. In order for gene-transfer to occur, the following events must take place: outcrossing and introgression. First outcrossing and successful fertilization of a non-transgenic ovule by pollen carrying the transgene must occur to produce a hybrid. So, outcrossing is a prerequisite to gene-transfer. The rate of outcrossing is dependent on the type of pollination and breeding system of the transgenic species. Creeping bentgrass has the following characteristics: small flowers, small or lacking perianth, no chemical attractants, anthers and stigmas exposed, production of copious amounts of small pollen grains and is wind pollinated [Kjellsson et al., 1997]. The breeding system of creeping bentgrass is predominantly cross pollinated and usually highly self incompatable (or self-sterile) [Björkman, 1960]. Both wind pollination and out-crossing would indicate a high outcrossing potential. Ellstrand and Hoffman [1990] reported a colonial bentgrass (*Agrostis capllaris*) gene flow of >1% at 8,000 m (26,304.64 ft) estimated by paternity analysis.

The second event for gene-transfer to occur is the introgression of the transgene. Introgression or introgressive hybridization occurs when outcrossing results in a stable incorporation of the transgene into the non-transgenic population. Of course, the rate of introgression is expected to be significantly higher in intra-specific hybrids than in inter-specific and inter-generic hybrids. The stability of the latter two groups of hybrids will depend on longevity (annual vs. perennial) or persistence, and cytogenetic behavior of the hybrids. Perennial hybrids will persist longer, increasing the possibility of producing progeny through backcrossing or chromosomal

doubling. Polyploidy also increases the chance of some fertility in the hybrid through homologous genomes shared between different taxa and so would allow the possibility of backcrossing and possibly result in fertile progeny.

Creeping bentgrass is cool season grass used for high quality putting greens, fairways, bowling clubs, and for closely cut lawns. The first transgenic turfgrass species to be requested for deregulation by APHIS will be a creeping bentgrass transformed for herbicide resistance since it is one of the highest valued turfgrass seed crops on the market.

However, successful commercialization depends on the crop being deregulated by USDA-APHIS, which requires a description of the known and potential differences from the unmodified recipient organism that would substantiate that the regulated article is unlikely to pose a greater plant risk than the unmodified organism from which it was derived, including but not limited to: 1) plant pest risk characteristics; 2) disease and pest susceptibilities; 3) expression of the gene product; 4) new enzymes; 5) changes to plant metabolism; 6) weediness of the regulated article; 7) impact on the weediness of any other plant with which it can interbreed; 8) agricultural or cultivation practices; 9) effects of the regulated organism on non-target organisms; 10) indirect plant pest effects on other agricultural products; 11) transfer of genetic information to organisms with which it cannot interbreed; and 12) any other information which the Administrator believes to be relevant to a determination (Regulation 7CFR340.6C(4)).

Creeping bentgrass is an out-crossing and wind pollinated, stoloniferous, perennial species. "Oregon produces nearly all of the bentgrass seed grown in the United States. Predominantly a Willamette Valley crop, bentgrass seed is exported in large quantities to Europe and also to the central and northern states for use in turf mixtures. This grass is widely used on golf courses throughout the world" [Young et al., 1997]. During the 1999 season, bentgrass was grown on 11,602 acres: 7,296 acres of creeping bentgrass; 4,300 acres of colonial and dryland bentgrass; and 83 acres of velvet bentgrass [OSCS, 1999]. In 1999, Oregon produced 4,778,000 lbs. of creeping bentgrass and 1,596,000 lbs. of colonial and dryland bentgrass [Young, 2000].

Creeping bentgrass has the potential to be the first perennial (stoloniferous), wind pollinated, outcrossing transgenic crop to be grown adjacent to naturalized and native populations of cross-compatible perennial relatives and native species. These are traits that can increase the risk of outcrossing, persistence, and introgression of alien genes into an adjacent population. However, the bulk of most of the risk assessment work conducted on transgenic plants has been on annual and/or self-pollinating crops. The potential risks from the commercialization and large-scale seed production of these types of transgenic crops is unknown. Research must be conducted to adequately assess the ecological and economic risks associated with introducing transgenes in a seed production situation. As increased research progresses in producing transgenic creeping bentgrass and as the release of a genetically engineered bentgrass cultivar draws closer, questions regarding pollen flow and gene introgression into local populations must be answered to insure environmentally and agriculturally safe introduction of transformed products.

Agrostis stolonifera has been documented as forming natural hybrids with the following species: 1) *A. canina*; 2) *A. capillaris*; 3) *A. castellana*; 4) *A. gigantea*; 5) *A. mertensii* Trinius; and 6) *A. vinealis* [Murbeck, 1898; Weber, 1920; Fouillade, 1932; Philipson, 1937: Stuckey and Banfield, 1946; Paunero, 1947; Davies ,1953a,b; Jones, 1953, 1956a,b,c; Bradshaw, 1958; Bradshaw, 1959; Björkman, 1960; Widén, 1971; Suckling and Forde, 1978; Bradshaw, 1975; Tutin, 1980; Edgar and Forde, 1991; and Forde, 1991]. Some of the interspecific hybrids are so common in nature that they have been given scientific names. The following interspecific hybrids have been formally named: 1) *A.* x *murbeckii* Fouillade (*A. stolonifera* x *A. capillaris*); and 2) *A.* x *bjoerkmanii* Widén (*A. capillaris* x *A. gigantea*)

A few studies have been published on artificial interspecific hybridization in *Agrostis*. The following experimental interspecific crosses have been made:

1) creeping bentgrass x *A. vinealis* [Björkman, 1960; Jones, 1956a, 1956b]. The seed set and pollen fertility were poor. At least 7 chromosomes remained unpaired in all cells; the maximum pairing was $1_{IV}\,5_{III}\,1_{II}\,7_{I}$. One genome of *A. stolonifera* showed partial homology with those of *A. vinealis*.
2) creeping bentgrass x *A. capillaris* (syn =*A. tenuis* Sibth.) [Björkman, 1960; Edgar and Forde, 1991; Davies, 1953a,b; Jones, 1956b, 1956c]. The seed set and pollen fertility were poor in one study, but *Agrostis capillaris* x *A. stolonifera* hybrids had 41.0% pollen fertility in another study. These hybrids were stoloniferous, vigorous, and apparantly long-lived in pastures [Forde, 1991]. *Agrostis stolonifera* and *Agrostis capillaris* probably have one ancestral diploid type in common and show good homology in one pair of their genomes and partial homology between the other one. The inter-genome pairing of *A. capillaris* is responsible for the fluctuation in pairing in the hybrids, which was also responsible for the fluctuation in pollen fertility [Jones, 1956b].
3) creeping bentgrass x *A. gigantea* Roth [Davies, 1953a,b; Jones, 1956c]. The seed set was 19.9 seeds/panicle and pollen fertility was 0 to 30% [Jones, 1956c]. The stable pairing and constant occurrence of seven univalents suggested that they constituted two pairs of highly homologous genomes, and the third show little homology with the others; the latter genome was possibly derived from an unknown ancestor. Davies [1953a] reported that the F_1 hybrids were quite fertile.

4) *A. capillaris* x *A. gigantea* [Davies, 1953a,b; Jones 1956c]. The seed set of the hybrids was 39.3/panicle and pollen fertility was 41 to 55%. The F_1 hybrids were as fertile as their parents on the basis of number of seeds per panicle under conditions of open pollination. The stable pairing and constant occurrence of seven univalents suggested that they constituted two pairs of highly homologous genomes, and the third showed little homology with the others; the latter genome was probably derived from the hexaploid parent (*A. gigantea*). The F_2 hybrids (inter-pollination of F_1's) had a pollen fertility of 53.3 to 62.8%. Backcrosses of the F_1 with A. capillaris (*A. capillaris* x *A. gigantea*) x *A. capillaris*) had a pollen fertility of 20.6 to 75% [Jones, 1956c]. Davies [1953a] reported that the F_1 hybrids were quite fertile.
5) *A. capillaris* x *A. vinealis* and (*A. capillaris* x *A. vinealis*) x *A. vinealis* [Jones, 1956b]. The F_1 hybrids between *A. capillaris* and *A. vinealis*, the F_2 generation, and backcrosses of the F_1's with *A. vinealis* were as fertile as the parents and were indistinguishable from *A. vinealis* at meiosis. The quadrivalent pairing sheds light not only on the interspecific relationships but also on the nature of *A. capillaris* itself. The purely bivalent pairing in *A. capillaris* disguised the fact that its genomes showed appreciable homology.
6) *A. castellana* x *A. capillaris* [Forde, 1991]. The hybrids were as fertile as the parents. The pollen fertility was 92.3%. The 11 F_1 hybrids were allowed to interpollinate with *A. capillaris* and *A. castellana* collections. The resulting progenies were vigorous and highly fertile, and displayed various combinations of the parental characters.

The hybrids were reported to have varying degrees of fertility, from sterile to as fertile as the parents. As would be expected, various genotypes differed in fertility, which mandated the use of multiple genotypes in crossing experiments. Most of the hybrids were vigorous regardless of fertility. The hybridization studies of Davies [1953a,b], Jones [1956a,b,c], Björkman [1960], and Forde [1991] clearly demonstrated that *A. canina*, *A. capillaris*, *A. castellana*, *A. gigantea*, *A. stolonifera*, and *A. vinealis* form a complex of interpollinating, cross-compatible species that readily cross when the species are sympatric (Fig. 1). *Agrostis stolonifera* (C_2C_2SS) is a allotetraploid, in which both of its genomes are highly homologous with two genomes of *A. gigantea* ($C_1C_1C_2C_2SS$). *Agrostis stolonifera* and *A. capillaris* ($C_1C_1C_2C_2$) have one genome in common. *Agrostis capillaris* is a segmental allotetraploid, in which both of its genomes are highly homologous with two genomes of *A. gigantea* ($C_1C_1C_2C_2SS$). Davies [1953b] concluded that both *A. capillaris* and *A. stolonifera* have something in common with *A. gigantea*, and they were involved in its evolution, or they at least have common origins with it. Widén [1971] also hypothesized that *A. gigantea* may have arisen from the fusion of C_1C_2S gametes of *A.* x *murbeckii* (the interspecific hybrid between *A. stolonifera* and *A. capillaris*). *Agrostis capillaris* also has a close chro-

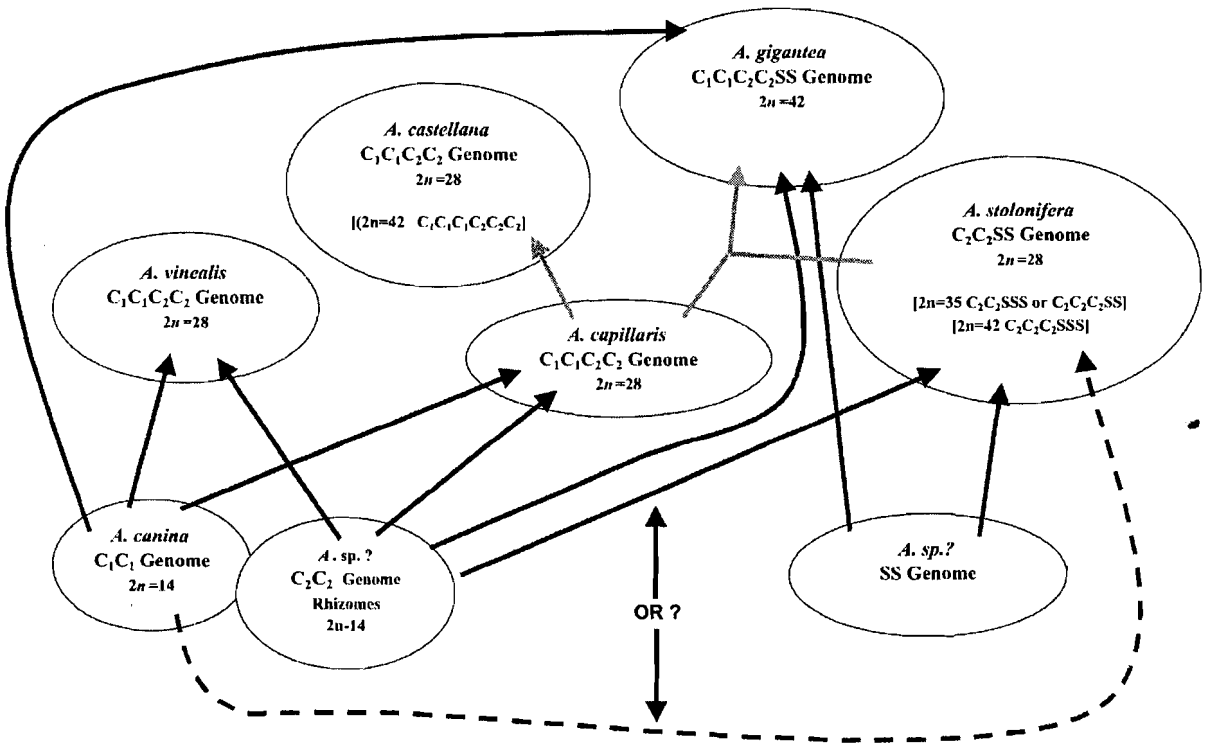

Figure 1. Genomic phylogeny of *Agrostis stolonifera* complex.

mosomal relationship with *A. vinealis* and *A. castellana*. *Agrostis vinealis* possibly evolved through introgression of a non-rhizomatous autotetraploid of *A. canina* with a rhizomatous plant of an *A. capillaris* type.

Intergeneric hybrids have also be reported between *Agrostis* and *Polypogon* [Björkman, 1960]. *Polypogon* species will hybridize with creeping bentgrass: 1) *Polypogon monspeliensis* (L.) R. Desfontaines, the hybrid between these two taxa occurs frequently enough that taxonomists have given it a name, x *Agropogon littoralis* (J.E. Smith) C.E. Hubbard; 2) *Polypogon viridis* (Gouan) Breistr., named x *Agropogon robinsonii* (Druce) Meldris & D. C. McClint.; 3) *P. fugax*; [Björkman, 1960]. Tutin [1980] hypothesized that *Agrostis parlatorei* Breistr. and *A. moldavica* Dobrescu & Beldie are perhaps derived through the hybridization of *A. castellana* and *Polypogon viridis*.

This information is relevant, because several of these *Agrostis* species occur in the Willamette Valley. "Information on the amount of out-crossing among *Agrostis* species does not exist for turfgrass seed production and managed turfgrass areas" [Johnson et al., 1999]. A total of 19 species have been documented in Oregon [Harvey, In Prep.]. Of the 19 species, 12 are reported to occur in the Willamette Valley and can be found in bentgrass seed production areas. Six of the 12 are naturalized species: *A. canina*, *A. capillaris*, *A. castellana*, *A. stolonifera*, *A. gigantea*, and *A. vinealis*. These six species are not only used commercially, but also are considered weeds in other crops. Of the remaining 12 species that are native to the area, the following six are known to occur in the Willamette Valley: *A. diegoensis* Vasey, *A. exarata* Trinius, *A. hallii* Vasey, *A. longiligula* A. Hitchcock, *A. oregonesis* Vasey, and *A. scabra* Willdenow. Carlbom [1966] in a biosystematic study of nineteen species of *Agrostis*, native to the Pacific states and all belonging to sect. *Trichodium* (Michx.) Trinius, reported on the reproductive biology of some of these species by conducting experiments on seed set in controlled self-pollinations. He reported that *A. exarata* was autogamous (self-compatible); *A. diegoensis*, *A. hallii*, and *A. oregonesis* were allogamous (self-incompatible or cross fertilized); *A. scabra* was both, depending on ecotype; and *A. longiligula* was not tested. Putative natural hybridization and introgression were observed between *A. diegoensis* and *A. hallii*; *A. diegoensis* and *A. pallens*. The outcrossing of these species with the six naturalized species was not studied.

Three species of *Polypogon* are also documented in Oregon: *P. monspeliensis* (L.) Desfontaines, *P. interruptus* Kunth, and *P. viridis* (Gouan) Breistroffer.

This objectives of this research were to study: 1) intra-specific gene movement; and 2) interspecific gene introgression and stability of the hybrids. The use of transgenic plants offers a unique opportunity to test and quantify pollen movement distances for creeping bentgrass using a dominant marker system.

MATERIALS AND METHODS

Transgenic Creeping Bentgrass

The transgenic plants that were used in this study were transformed by microprojectile bombardment at Michigan State University, by Dr. C.-A. Lu [1996] in Dr. Mariam Stricklen's lab. The genes were introduced on a commonly used plasmid vector, pUC #19. The plants were genetically engineered for tolerance to the herbicide glufosinate through the expression of the *bar* gene derived from *Streptomyces hygroscopicus*, which encodes the enzyme phosphinothricin acetyl transferase (PAT). PAT inactivates the herbicide phosphinothricin (PPT) by acetylation, which converts the active compound to a herbicidally-inactive product [Murakami et al., 1986; Thompson et al., 1987; Vasil, 1996]. PTT is the active ingredient in glufosinate or bialaphos. Glufosinate is a non-selective herbicide that inhibits the enzyme glutamine synthase. The inhibition of this enzyme allows ammonia levels to increase to phytotoxic levels, leading to cell death [Tachibana et al., 1986a,b; Devine et al. 1993]. Because higher plants cannot naturally convert these herbicides to inactive products, transformation of crops with the bar gene may allow for complete weed control and result in little damage to the transformed plant. Moreover, the presence of this pathway in bacteria, but not in plants, is indicative of a low potential for the development of resistance [Smeda and Vaughn, 1997]. The noncoding DNA regulatory sequences that control expression of the introduced genes are the CaMV 35s promoter and the nos terminator from *Agrobacterium tumefaciens* T-DNA. A proteinase inhibitor gene (*pin-2*) derived from *Solanum tuberosum*, was also part of the gene construct, but this gene has not been shown to affect the phenotype of the bentgrass.

Of the 60 transformants recovered, all were sterile (either did not flower or if flowering culms were produced, they failed to produce viable reproductive organs), except one. This plant flowered and produced viable pollen and ovaries and was successfully crossed with Seaside II creeping bentgrass in the greenhouse out Michigan State University by Dr. Scott Warnke. Approximately 1,000 progeny were recovered. These prospective transformants were screened for herbicide tolerance with a 6X label rate (label rate: 24 oz/ac) of Ignite®, resulting in 480 herbicide tolerant transformed seedlings. These plants were sent to Oregon and 286 were planted into a nursery. The other 194 plants died en route to Oregon due to freezing temperatures.

Lee et al. [1996] conducted herbicide tests on transgenic creeping bentgrass with Herbiace®, a commercial formulation of bialaphos released in 1978 by Meijii Seika Kaishya, LTD., Tokyo. The herbicide rates were established with control plants, and were applied at the commercial rate of 0.75 lb AI/ac (1 x the field rate). The transgenic plants were resistant to 5x the field rate of Herbiace®. Lee and Day [1996] reported that one-

Table 1. Description of 1998 transects.

Transect Acronym	Description of Transect	Number of plants planted	Number of plants actually harvested
LC	272.5 ft (83.1 m) circle with 100 ft (30.48 m) spacing	17	13
SC	109 ft (33.2 m)circle with 50 ft (15.24 m) spacing	14	13
NET	NE line from edge to 244 ft (74.4 m). 10 ft (3.05 m) spacing for first 120 ft (36.6 m) and then 20 ft (6.1 m) spacing	17	17
SWT	SW line from edge to 300 ft (91.4 m). 10 ft (3.05 m) spacing for first 120 ft (36.6 m) and then 20 ft (6.1 m) spacing	21	21
RNE	NE Transect through rye. 5 ft ((1.52 m) spacing	9	5
RSW	SW Transect through rye. 5 ft (1.52 m) spacing	9	6
RI	Transect inside rye border 10 ft (3.05 m) spacing	22	22
Row 10	Row 10 is on N side in transgenic nursery, Non-transgenic plants	32	29
RM	Transect in the rye border. 6 ft (1.8 m) inside the rye and 10 ft (3.05 m) spacing	42	40
RO	Transect around the outside of the rye border, 10 ft (3.05 m) spacing	54	45
Total		237	211
Agrostis spp.	*Agrostis* spp. planted into nursery	13	13
TOTAL		250	224

month-old bentgrass plants, transformed with the bar gene, were unaffected by a 5x field rate of Herbiace® (2 mg/mL). No control plants survived the herbicide treatment after 10 days, while the transgenic plants were undamaged and as healthy as the control plants. Zhong et al. [1996] sprayed 38 independent transgenic lines with 1.2% Ignite® (label rate: 2.4 g/L of glufosinate ammonium, 1.50 L/ha). This rate killed the control plants within 10 days and 37 of the 38 transgenic lines showed no obvious damage. Zhong et al. [1996] also found from evaluating the herbicide resistance levels of three independent events that the levels of PAT activity were different. The LD_{50} values (lethal dose that caused 50% of plant death) of the three tested transgenic lines were about 20, 30, or 40 times higher that that of non-transgenic plants. These tests showed that the labelled rate of glufosinate herbicides are sufficient to kill non-transgenic plants and demonstrated that the transgenic plants have a high level of resistance to PPT.

Non-transgenic Creeping Bentgrass

The non-transgenic creeping bentgrass cultivar used in this study was Penn *A-1*. This cultivar was chosen because the plants were similar in age and maturity to the transgenic plants. Creeping bentgrass has a maturation requirement to reach optimum flower production and must be vernalized. Generally plants begin flowering their second year after vernalization. The plants were taken from a two year old production field (planted in 1997), which was the same age of the transgenic plants. The field was 4 mi south of the study site. New plants for the transects were dug each year for the study.

The Penn A-1 plants were evaluated for tolerance to glufosinate to establish the rate for screening seedlings containing the bar gene. Five different non-transgenic genotypes (including Penn A-1) of creeping bentgrass and one genotype of colonial bentgrass were screened with Finale®. Approximately 2,000,000 seedlings were tested for each genotype, with two of the genotypes tested twice (Penn A-1 and Penn A-2). A total of ± 14,000,000 seedlings of creeping bentgrass and 2,000,000 seedlings of colonial bentgrass were sprayed twice with a 5.7 L/.4 ha (6 qts/ac) rate of Finale® and no survivors were found within 14 days. Transgenic bentgrass seedlings were not damaged with this rate.

Experimental Design Of Transects for Infra-specific Gene Flow

Approximately 250 non-transgenic bentgrasses were planted in transects around the 286 transgenic bentgrass plants [36 x 128 ft nursery (≈ 0.1 ac or 0.04 ha)] in 1998 and 1999. This ratio of transgenic to non-transgenic plants was sufficient to detect the spread of the bar gene. Gliddon [1999] pointed out that the vast majority of pollen dispersal studies involving marker genes have the transgenic organism as a small minority of the total organisms in the design, making it difficult to detect the spread of the marker gene in relation to the probability of recovering the non-marked gene. If the marker is represented by 1% of the total organisms, even if its spread is uniform across the entire experimental area, it will only be recovered in 1% of samples. "This fault of experimental design could well account for the very small distances that have been reported for the spread of GMO's [e.g. Scheffler et al., 1993]" [Gliddon, 1997].

In 1998, the following transects were established (Table 1, Figure 2): 1) two circles around the nursery at 109 (33.2 m) and 272.5 ft (83.1 m) with plants spaced at 50 ft (15.24 m) and 100 ft (30.48 m), respectively; and 2) two line transects aligned with prevailing winds (NE) with one transect NE and the SW of the transgenic nursery. The NE transect extended 244 ft (74.4 m) from the NE edge of the nursery and the SW transect extended 300 ft (91.4 m) from the SW edge of the nursery. Plants in the line transects were spaced 10 ft (3.048 m) apart for the first 120 ft (36.6 m), then spaced 20 ft (6.1 m) after. The initial length of the transects was based on Oregon Seed Certification isolation distances, which is 165 to 300 ft (50.3-91.4 m) (depending on field size) for certi-

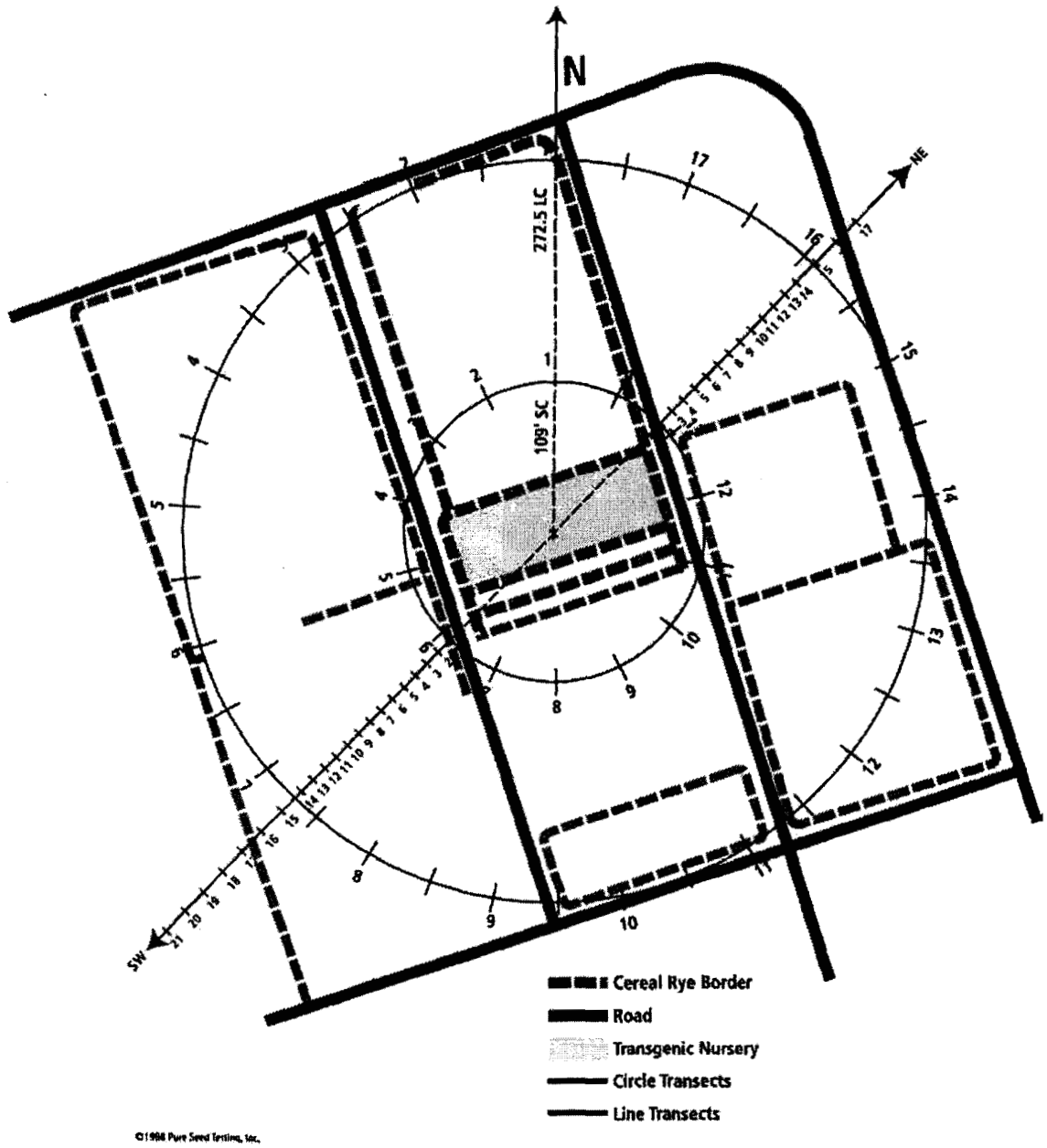

Figure 2. Overview of transects for pollen travel study.

fied seed production. To test the effectiveness of cereal rye (*Secale cereale* L.) as a pollen barrier, non-transgenic plants were planted around the inside of the transgenic nursery, six feet (1.8 m) inside the cereal rye, on the outside of the cereal rye border, and through the NE and SW corners of the cereal rye. One hundred sixty-eight plants were placed in or around the cereal rye border.

In the 1999 the experimental design was similar to that of 1998 with the exception of the following (Figure 3): 1) two additional line transects (SE and NW), orthogonal to the prevailing winds were added; 2) the length of the line transects were increased, where possible, to the following: a.) SW transect, 978 ft (298.1 m); b.) NE transect, 268 ft (81.7 m); c.) SE transect, 612 ft (186.5 m); and d.) NW transect, 319 ft (97.2 m). Non-transgenic were also planted around the transgenic nursery both years; 3) Plants in the line transects were spaced 20 ft (6.1 m) apart for the first 300 ft (91.4 m) and then every 10 ft (3.048 m) apart after; 4) the plants in the 272.5 ft (83.1 m) radius transect were spaced every 50 ft (15.24 m), not 100 ft as in the previous year; 5) only 20 non-transgenic plants were planted around the outside of the transgenic nursery – 5 plants on each side; and 6) Row 10, the non-transgenic row of plants in the transgenic nursery, was the only non-transgenic plants in the nurs-

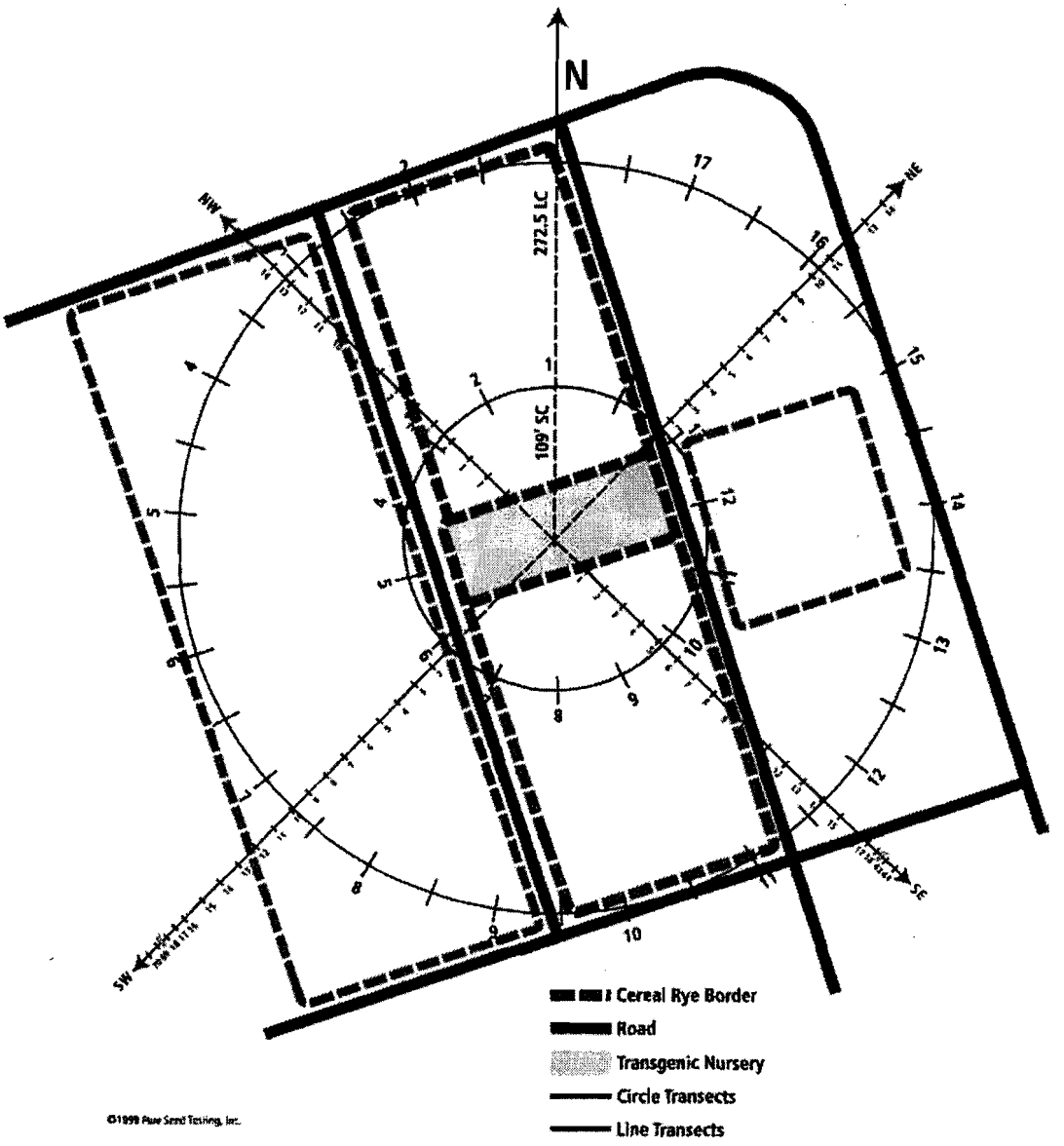

Figure 3. Overview of transects for pollen travel study.

ery.

Non-transgenic plants were planted into the transects during February and March of each year. Once the plants finished flowering, inflorescences were enclosed in hybridization bags. Any remaining, unbagged, inflorescences were cut and burned to prevent any contamination. The inflorescences were then harvested and the non-transgenic plants were killed with herbicide (Roundup®) and then burned; they were replanted each year. The removal of non-transgenic plant prevented the contamination of this plant from any transgenic seed that may have been produced, fallen to the ground, germinated and grew in with the non-transgenic plant. If this occurred, then this plant would be producing transgenic pollen along the transect.

The harvested inflorescences were dried in the greenhouse. Once dry, seeds from each non-transgenic plant were planted and screened in greenhouse for herbicide resistance; 1000+ seeds were planted in order to get 1,000 seedlings to be screened. The seedlings were sprayed 2 to 3 times with Finale™ or Rely® (which has now replaced Finale™) once they reached the 3 to 4 leaf stage, with a rate of 5.7 L/.4 ha (6 qts/ac). This rate is listed for plants 8 in (20.32 cm) or taller. This rate has been tested on five different non-transgenic genotypes of creeping bentgrass and one genotype of colonial bentgrass. Approximately 2,000,000 seedlings per genotype were tested, with two of the genotypes tested twice. A total of ± 24,000,000 seedlings of creeping bentgrass and 2,000,000 seedlings of colonial bentgrass were sprayed twice with a 5.7 L/.4 ha (6 qts/ac) rate of Fi-

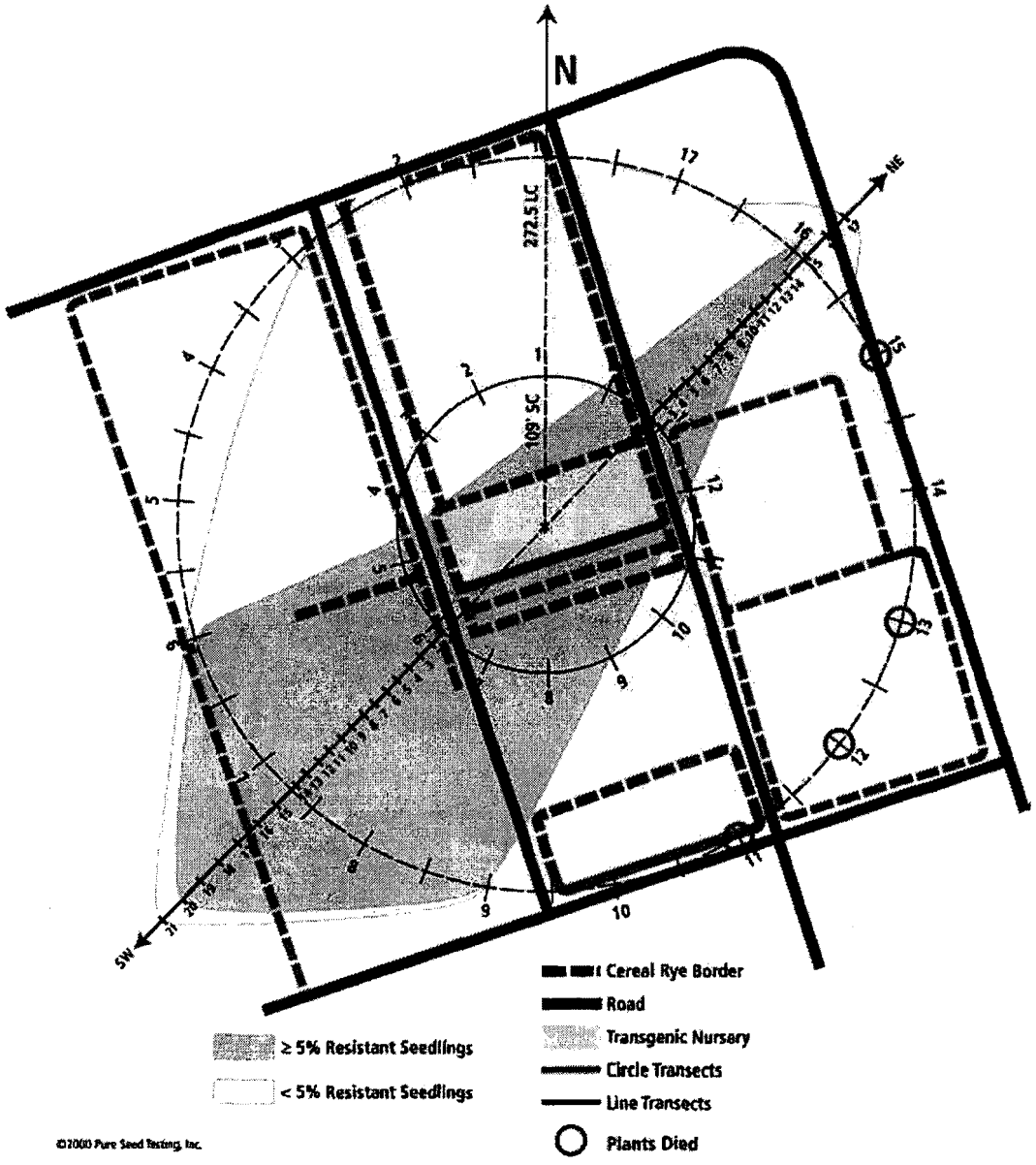

Figure 4. 1998 transgenic bentgrass gene flow

nale™ with no survivors. Whereas, transgenic bentgrass plants were not damaged with this rate.

Screening seedlings with the high labelled rate of glufosinate (5.7 L/.4 ha (6 qts/ac) for Finale™) will sufficiently kill the plants and only those plants with the functioning bar gene can survive that rate, even though a low level of resistance to glufosinate is sometimes seen.

The number of survivors and the number of dead were counted. A sample of the survivors were sent to a biotechnology lab to confirm the presence of the bar gene using PCR and Southern Blot analysis. Once the percentage of resistant to non-resistant seedlings was determined, the data were then analyzed with Graphpad Prism® non-linear regression software. The curve that best fit the data was a 'Top to Zero One Phase Exponential Decay' Model. This allowed for the prediction of the percent recovery of the transgene over distance.

Inter-specific Gene Flow

Plants were placed in the transgenic nursery next to plants with a similar phenology. Accessions of *A. canina*, *A. capillaris*, *A. castellana*, *A. curtissii*, *A. gigantea*, in 1998, and *A. pallens* and *A.* sp. eastern Oregon, in 1999, were planted in the transgenic nursery prior to flowering and allowed to interpollinate. Seed was collected from these accessions, planted, and screened for herbicide resistance as described above.

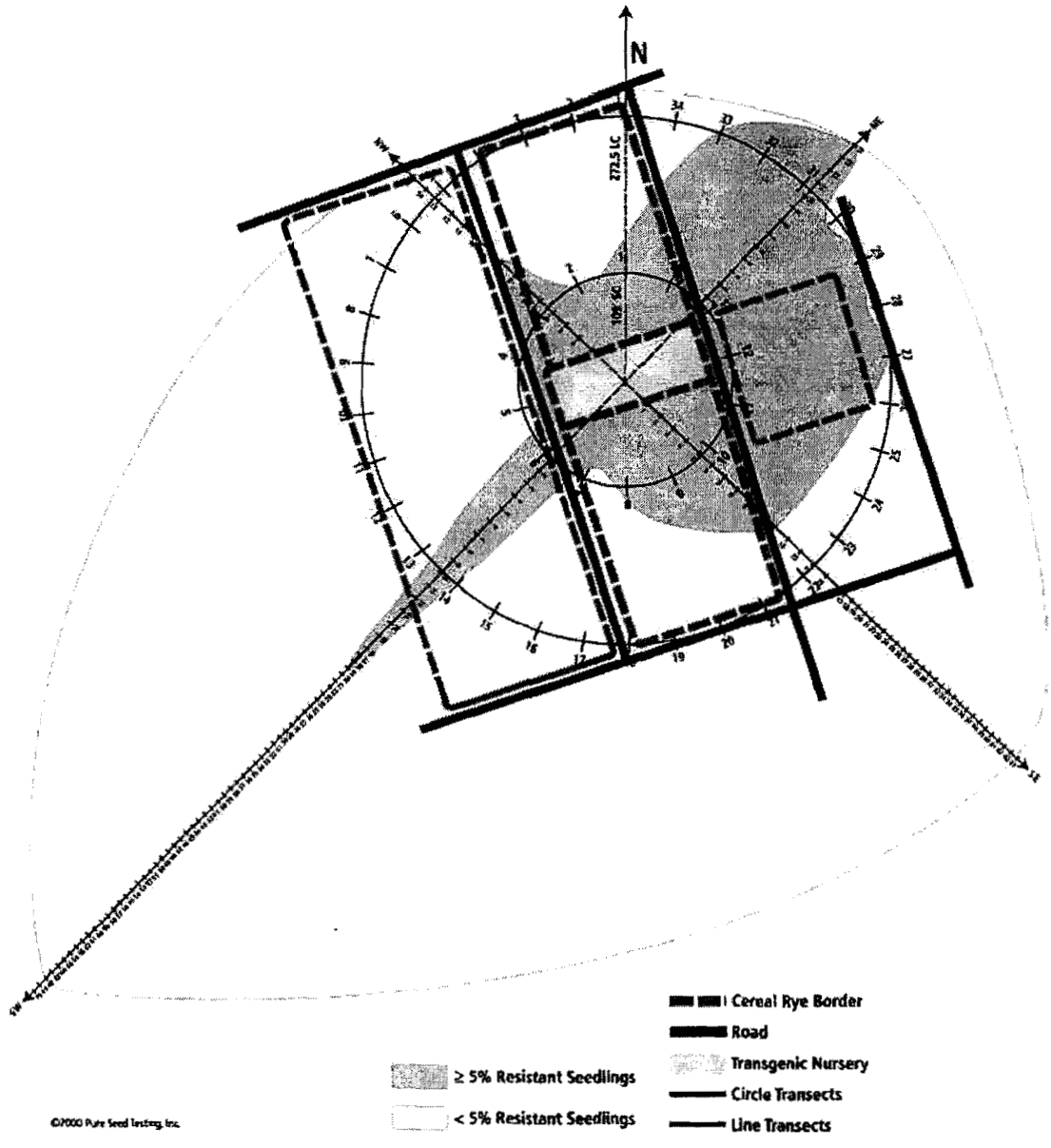

Figure 5. 1999 transgenic bentgrass gene flow.

RESULTS AND DISCUSSION

Intra-Specific Gene Flow

In 1998, >0.02 % transgenic plants were recovered at the ends of both the NE (244 ft (74.4 m) and SW (300 ft (91.4 m) transects, as well as, from most of the points along the circle transects (Figure 4). In 1999, >0.02 % transgenic plants were recovered at the following distances: 1)NE transect, 268 ft (end of transect); 2) SW transect, 958 ft; 3) NW transect, 319 ft; and 4) SE transect, 612 ft from edge of transgenic nursery (Figure 5).

The distribution of the data was determined to be strongly leptokurtic and not normally distributed, which is congruent with other pollen distribution data (as discussed in the Introduction). The data were then analyzed using non-linear regression, since the goal of regression was to find a curve that best predicted Y from X, an exponential decay model was determined to fit the data well. Using this model, the following distances were predicted for transgenic pollen introgression to the 0.02 % level. In 1998, along the SW transect, transgenic pollen traveled 3,500 ft (1,066.8 m) and along the NE transect, it traveled 4,296 ft. (1,309.4 m) (Figures 6-7). In 1999, the transgenic pollen was estimated to have trav-

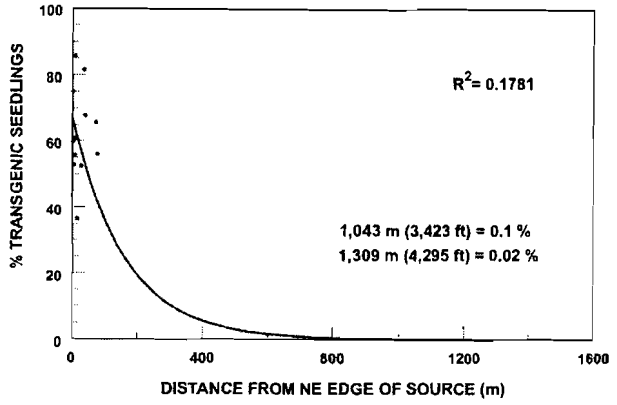

Figure 6. 1998 Transgenic pollen flow along NE transect.

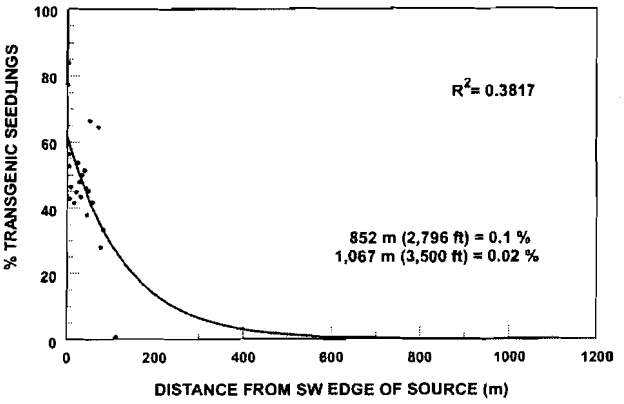

Figure 7. 1998 Transgenic pollen flow along SW transect.

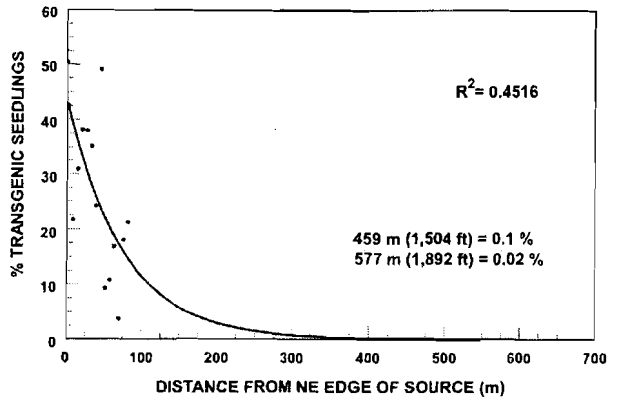

Figure 8. 1999 Transgenic pollen flow along NE transect.

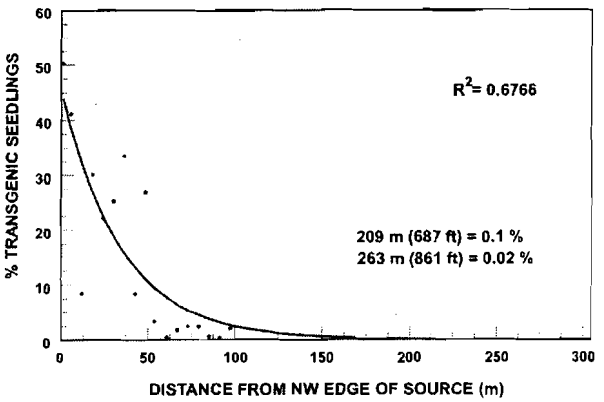

Figure 9. 1999 Transgenic pollen flow along NW transect.

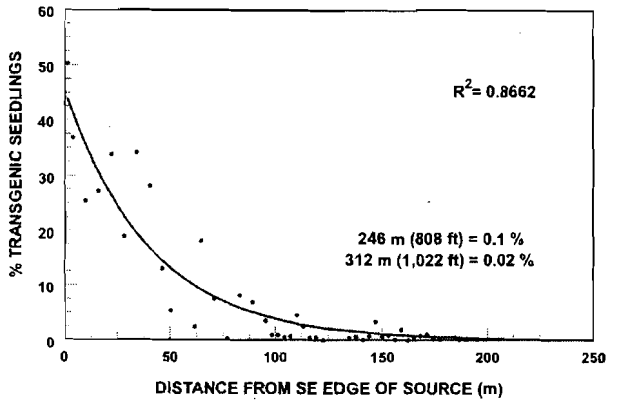

Figure 10. 1999 Transgenic pollen flow along SE transect.

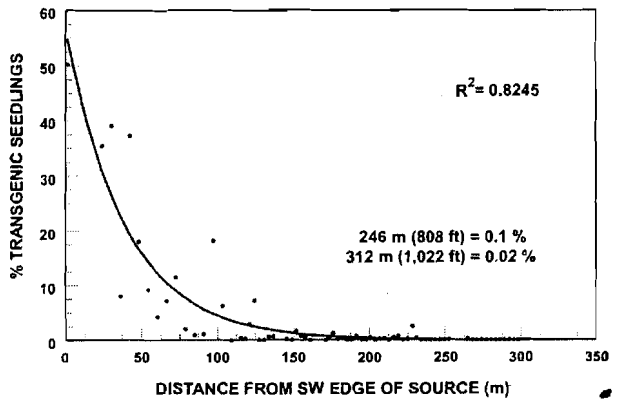

Figure 11. 1999 Transgenic pollen flow along SW transect.

eled 1,022 ft (331.5 m) to the SW, 1,892 ft (575.1 m) to the NE, 861 ft (262.4 m) to the NW, and 1,022 ft (331.5 m) to the SE (Figures 8-11). A diagrammatic representation of predicted pollen flow based on the exponential decay model results for 1998 and 1999 pollen flow data can be seen in Figures 12 and 13.

The higher pollen flow in 1998 was also seen in adjacent nurseries. Seed was sampled from a nursery 638 ft (194 m) S of the transgenic nursery. Approximately two million seeds (160 g) were planted and three applications of Finale® were applied to the seedlings. Three hundred fifty-four seedlings were found to be resistant. Five plants were sent to a lab to confirm the presence of the bar gene. Seeds are also sampled from a Penn A-1 breeder seed field 1,417 ft (432 m) SW of the transgenic nursery and a Penn A-2 field just to the SE . Two million seeds were planted of each cultivar and two to three applications of Finale® were applied to the seedlings. After the second application of herbicide, no Penn A-2

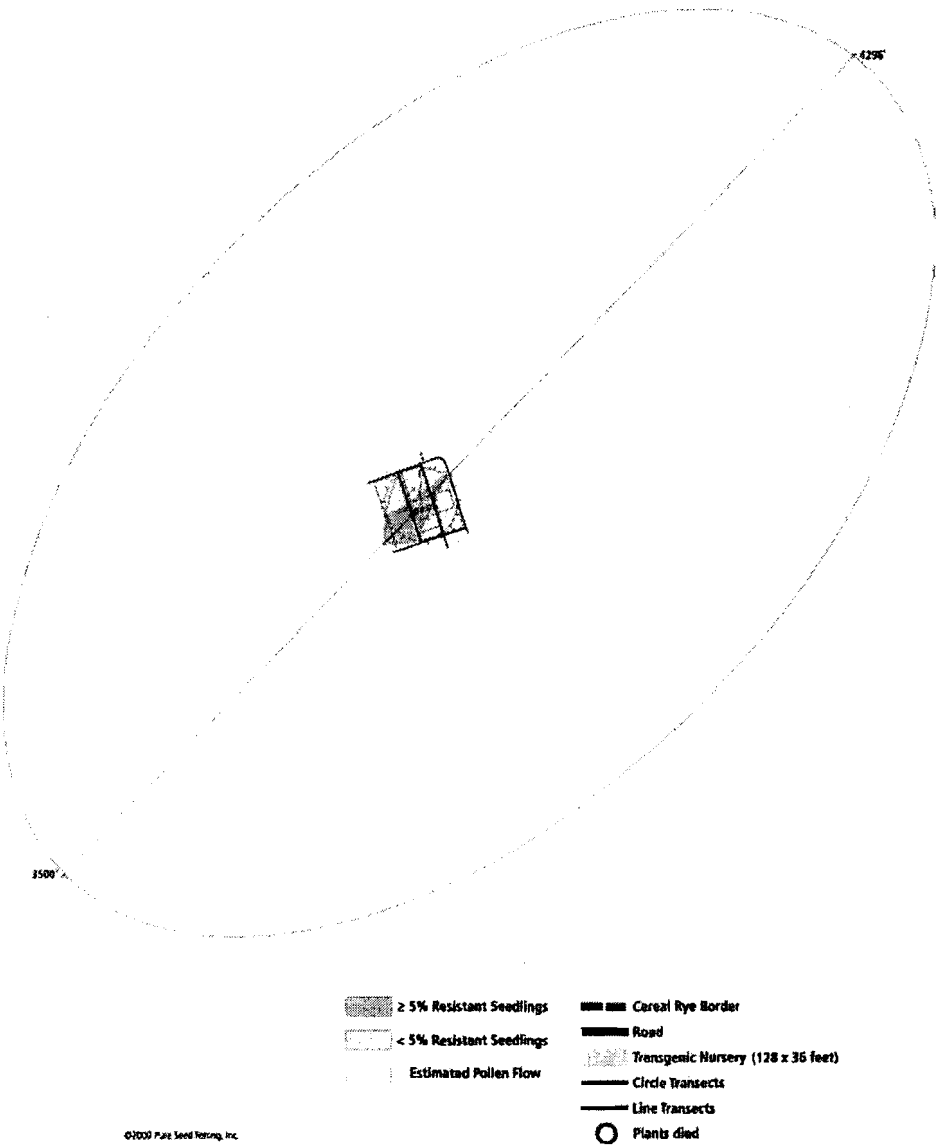

Figure 12. 1998 transgenic bentgrass gene flow.

seedlings survived; however Penn A-1 had survivors. Three plants survived and vegetative material was sent to a lab to confirm the presence of the bar gene with Southern Blot analysis. The lab confirm that the surviving plants from the two nurseries did contain the bar gene.

The same two nurseries (Penn A-1 and OVN) were retested in 1999. Penn A-2 was not tested, because the nursery was destroyed during the fall 1998. Again, 160 g of seed was planted from each nursery and sprayed with 2 to 3 applications of Finale®. No seedlings survived from the Penn A-1 nursery and 459 seedlings were recovered from the OVN nursery. A sample of these possible transformants is currently being tested for the bar gene.

This not only demonstrated that pollen was viable for at least 1,400 ft (426 m), but also established successful competition with non-transgenic pollen in a field situation. The Penn A-1 nursery was 0.1 ac (0.04 ha) in size, similar to that reported by Giddings [2000] with perennial ryegrass.

Inter-Specific Gene Flow

The crossing experiments resulted in the introgression of the bar gene from creeping bentgrass into *A. canina*, *A. capillaris*, *A. castellana*, *A. gigantea*, *A. pallens* and *A.* sp. (eastern Oregon). The following are the results from the different species of *Agrostis* that were planted within the transgenic nursery in 1998 and 1999.

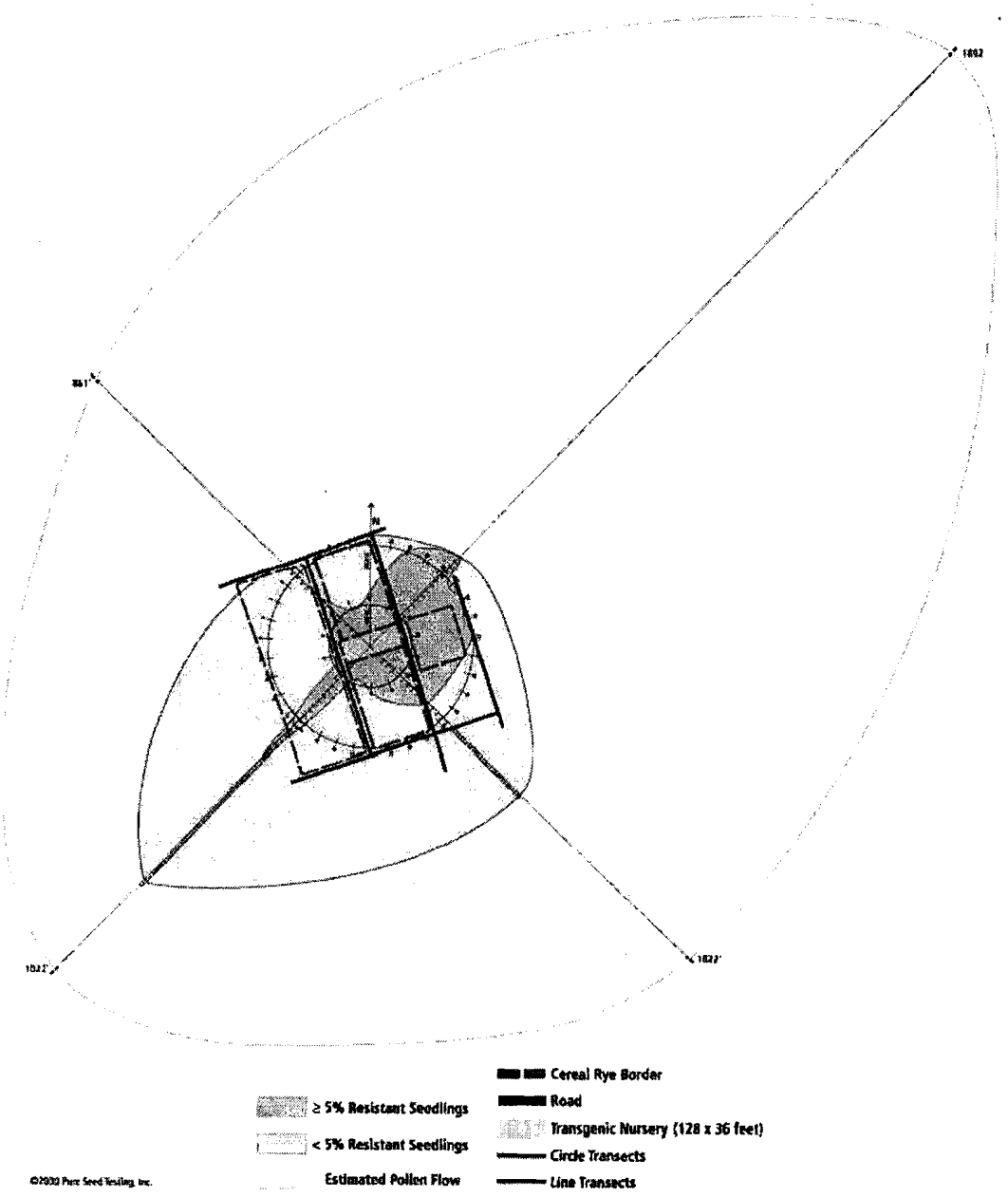

Figure 13. 1999 transgenic bentgrass gene flow.

Species	Origin or Code	Number of Survivors
A. canina	SR 7200	7
A. capillaris	9NC-20	13
A. capillaris	9NC-21	24
A. capillaris	9NC-78	0
A. castellana	New Jersey	51
A. castellana	Rhode Island	5
A. castellana	Oregon	32
A. curtissii	Portugal	0 (0 germination)
A. gigantea	Tennessee	1
A. gigantea	Tennessee	4
A. gigantea	Kentucky	5
A. gigantea	Kentucky	0
A. pallens	Oregon	4 (11.11 %)
A. sp.	Oregon	6 (15.8 %)

The results showed that the transgenic gene occurred not only between species known to cross with creeping bentgrass, but also with species native to Oregon. Future research will concentrate on the stability and fertility of the interspecific hybrids and include more native species in the study.

CONCLUSIONS

The results from the this study showed: 1) the transgenic bar gene can flow to other species of *Agrostis* (i.e. interspecific gene flow); 2) intraspecific gene flow in creeping bentgrass is possible for much longer dis-

tances than traditionally theorized; 3) the transgenic bentgrass plants were fertile and stable; and 4) the cereal rye was not an effective pollen barrier.

The distance that the creeping bentgrass pollen traveled was congruent with the documented gene flow from other wind pollinated and cross-pollinated species. Giddings [2000] determined that *Lolium perenne* pollen can easily travel a kilometer. Devlin and Ellstrand [1990], using a method of paternity analysis, reported a gene flow greater than 1 % at 8000 m distance in *Agrostis capillaris* (syn = A. tenuis). Gene flow from crop to weeds or feral populations via insect pollinators occurred up to one kilometer in: 1) radishes (*Raphanus sativus* L.) [Ellstrand et al., 1989; Klinger et al., 1991]; sunflowers (*Helianthus annuus* [Arias and Rieseberg, 1994; Whitton et al., 1997]; and squash (*Cucurbita* spp.) [Kirkpatrick and Wilson, 1988]. Thompson et al. [1999], studied gene flow in oilseed rape (*Brassica napus*) L. at two sites. At the first site 52 sites were selected between 0 to 4000 m from the nearest crop source (no transgenic plants were being grown at this study site). At each site, male-sterile bait plants were placed for a 14 day period and airborne pollen density measured. Pollination occurred at all sited and although oilseed rape pollen was recorded at all sites, the density declined rapidly with distance from the source. In a second study with sites 100 to 900 m from a transgenic oilseed crop, all sites were pollinated by a mixture of transgenic and non-transgenic sources.

Absolute containment of transgenes is undoubtedly often impossible. Genes can not only escape via the pollen, but also through seeds that are left in fields and lost during handling. Instead of asking whether absolute containment is possible, approaches that will maximize the level of containment should be the focus. The spread transgenes through the pollen to cross-compatible populations can be prevented by: 1) male sterility which will prevent development of pollen; 2) transformation of chloroplast, which will insure that the gene is only maternally inherited; 3) growing the crop in areas where no cross compatible populations exist; and 4) at the current pace of biotechnological discoveries and development, new technology, not even thought of today, will be developed in the future that could eliminate this problem altogether. The future of biotechnology is bright, unlimited and very exciting. This molecular revolution, like any other revolution that has occurred throughout human history, will go through growing pains. But through sound research to gather scientific data to make informed decisions and through public education of the perceived 'sci-fi' technology, the potential of this technology will be realized.

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Evidence for landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with *CP4 EPSPS* as a marker

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Sampling methods and results of a gene flow study are described that will be of interest to plant scientists, evolutionary biologists, ecologists, and stakeholders assessing the environmental safety of transgenic crops. This study documents gene flow on a landscape level from creeping bentgrass (*Agrostis stolonifera* L.), one of the first wind-pollinated, perennial, and highly outcrossing transgenic crops being developed for commercial use. Most of the gene flow occurred within 2 km in the direction of prevailing winds. The maximal gene flow distances observed were 21 km and 14 km in sentinel and resident plants, respectively, that were located in primarily nonagronomic habitats. The selectable marker used in these studies was the *CP4 EPSPS* gene derived from *Agrobacterium* spp. strain CP4 that encodes 5-enol-pyruvylshikimate-3-phosphate synthase and confers resistance to glyphosate herbicide. Evidence for gene flow to 75 of 138 sentinel plants of *A. stolonifera* and to 29 of 69 resident *Agrostis* plants was based on seedling progeny survival after spraying with glyphosate in greenhouse assays and positive TraitChek, PCR, and sequencing results. Additional studies are needed to determine whether introgression will occur and whether it will affect the ecological fitness of progeny or the structure of plant communities in which transgenic progeny may become established.

We developed sampling methods and describe results of a gene flow study that will be of interest to plant scientists, evolutionary biologists, ecologists, and stakeholders assessing the environmental safety of transgenic crops. Creeping bentgrass (*Agrostis stolonifera* L.) is one of the first wind-pollinated, perennial, and highly outcrossing transgenic crops being developed for commercial use. Unlike currently commercialized transgenic crops in the U.S., which have no synchronously flowering relatives in areas of commercial production, the cosmopolitan genus *A. stolonifera* has compatible relatives in a broad variety of habitats. The methods and results of using herbicide resistance as a selectable marker from a genetically modified (GM) crop to measure gene flow will be useful for assessing the potential for GM crops to transfer their novel genes to compatible relatives.

More data are available on gene flow from cultivated crops to other crops than from crops to resident (native, naturalized, or weedy) species (1). Typically, gene flow distances are reported on the scale of meters, much less often on the scale of kilometers. Maximum reported distance for gene flow between radish and wild radish (2) and between cultivated and wild sunflowers is 1,000 m (3); distances of 1,300 m have been reported between cultivated and wild squash (4). In an Australian study, crop-to-crop transfer distance of 3,000 m has been reported from source fields of nonGM herbicide-resistant canola to fields of herbicide-sensitive canola cultivars (5).

In this study, we present evidence that documents multiple instances at numerous locations of long-distance viable pollen movement from multiple source fields of GM creeping bentgrass. We used the *CP4 EPSPS* gene that encodes 5-enol-pyruvylshikimate-3-phosphate synthase from *Agrobacterium* spp. strain CP4 as a selectable marker to track gene movement. This gene confers resistance to glyphosate (*N*-phosphono methyl-glycine), the active ingredient in RoundUp herbicide (Monsanto, St. Louis, MO). Herbicide resistance as a result of expression of the engineered *CP4 EPSPS* gene was observed in seedling progeny of sentinel *A. stolonifera* and resident *Agrostis* spp. located at distances up to 21 km and 14 km, respectively, from the crop fields. Eight source fields totaling ≈162 hectares (ha) were located on an irrigated plateau above the Deschutes River in central Oregon. The fields were contained within a 4,453-ha GM bentgrass control district (http://arcweb.sos.state.or.us/rules/OARS_600/OAR_603/603_052.html; ref. 6) located ≈144 km east of commercial nonGM bentgrass seed production areas in Oregon's Willamette Valley. When the source fields of GM creeping bentgrass flowered for the first time during the summer of 2003, they presented a unique opportunity to use the *CP4 EPSPS* gene as a marker to quantify viable GM pollen movement and potential gene flow to compatible resident and sentinel plants located in areas beyond the crop source fields. Results presented here use multiple lines of evidence based on assays of seedlings germinated from seed harvested from sentinel and resident plants. These assays include tests in greenhouse settings for survival after spraying with RoundUp and tests for presence and expression of the *CP4 EPSPS* marker.

A. stolonifera is a cool season, wind-pollinated perennial grass used on golf courses around the world (7). It also is of interest as a forage crop (8), for phytoremediation of heavy metals in soils (9), and for water quality improvement by biofiltration (10). The taxonomically uncertain genus *Agrostis* is estimated to include >200 species worldwide (11, 12). In North America, 26 species of *Agrostis* are considered native, including 14 species found in Oregon (http://plants.usda.gov). *Agrostis* is found in riparian habitats, agronomic and urban settings, mountain meadows and woodlands, coastal sand dunes, fresh and salt water marshes, ditches, pastures, grasslands, and roadsides (13, 14). The small seeds of *A. stolonifera* (up to 6×10^6 per pound) are readily dispersed by wind, water, and animals (13, 15). Introduced and widespread in the U.S., *A. stolonifera* is sometimes considered an economic weed, e.g., as a volunteer in grass seed or other agronomic production fields and as a colonizer of nonagricultural habitats; it has been reported as weedy in Japan, Australia, New Zealand, Chile, Germany, Denmark, the United Kingdom, and Canada (16).

A. stolonifera is generally considered to be an obligate outcrosser (17); however, self-fertility also has been reported (18).

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Abbreviations: GM, genetically modified; ha, hectare.

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The species is most typically an allotetraploid (19, 20) and has cytotypes of higher ploidy (21). Naturally forming interspecific F_1 hybrids generally are low in fertility or sterile; in favorable habitats, some hybrids (e.g., F_1 hybrids of *A. stolonifera* and *Agrostis capillaris* L.) have been reported to out-compete both parents (22). There are few clear examples of F_2 hybrids (23) or of backcrosses of F_1 hybrids to a parental species (18). Although native or naturalized hybrids may be sterile, they can constitute a significant component of plant communities because of vegetative spread by means of stolons (24).

Field studies of hybridization between *A. stolonifera* and other species of *Agrostis* or between *A. stolonifera* and closely related *Polypogon* spp. (18, 25, 26) have produced similar findings on outcrossing ability. In a field study that included several hundred plants as sources of pollen from bentgrass engineered to be resistant to glufosinate herbicide, a gene flow distance of 298 m was reported (25). Natural hybrids of *A. stolonifera* have been reported with six other native species.: *Agrostis canina* L., *A. capillaris* L., *Agrostis castellana* Boissier and Reuter, *Agrostis gigantea* Roth, *Agrostis mertensii* Trinius, and *Agrostis vinealis* Schreber (www.essentialbiosafety.info/docroot/articles/02-281-009.pdf). A computer model (27) found that pollen dispersal and gene introgression would be limited at some sites and extensive at others, depending on local wind conditions.

Materials and Methods

Sampling Design. A sampling grid (Fig. 1) was designed to determine the extent of viable GM pollen flow based on the temporary deployment of 178 compatible *A. stolonifera* sentinel plants and the monitoring of naturally occurring compatible resident plants. Critical assumptions in the sampling design included a maximal pollen viability of up to 3 h (28) and prevailing winds of 10 km/h from the north and northwest (data are from the Pacific Northwest Cooperative Agricultural Weather Network weather data archive, see www.usbr.gov/pn/agrimet/) during the expected period and hours of anthesis (e.g., mid-June to early July, from 11 a.m. to 2 p.m.) of the source GM creeping bentgrass crop fields. Thirty locations with resident plants of *A. stolonifera*, 39 locations with resident plants of *A. gigantea*, and 10 locations with resident plants of *Polypogon monspeliensis* (L.) Desfontaines also were included in the study. Plants of *A. stolonifera* (experimental population no. 1 CRBP, Seed Research of Oregon, Corvallis, OR) cultivated in a field in the Willamette Valley of western Oregon were transplanted to 23-cm diameter pots and used as sentinel plants. Before their transport to central Oregon, each of the sentinel plants tested negative for *CP4 EPSPS* by the TraitChek immunological lateral flow test strip method (Strategic Diagnostics, Newark, DE). Each of the 69 resident *Agrostis* plants and the 10 *P. monspeliensis* resident plants were tested by using the TraitChek method to ensure that they were negative for the *CP4 EPSPS* protein that confers resistance to glyphosate. In mid-June, sentinel plants were deployed to field positions at times of day when anthesis from the source fields was considered unlikely (i.e., before 8 a.m. and after 6 p.m.). Additional steps taken to minimize incidental pollination of sentinel plants included bagging each plant during transit and distribution of the plants by geographic sector. Within sectors, the first plants that were put in place were those at the greatest distance from the perimeter of the control district; the last plants that were placed within a sector were those closest to the control district perimeter. In mid-July, after anthesis in the source fields had ended, panicles were bagged in the field. Bagged sentinel plants with bagged panicles and bagged panicles from resident populations were collected several weeks later. These measures allowed for *in situ* seed fill and for temporal separation with seed harvesting activities on the GM bentgrass fields. An additional precaution taken to prevent dissemination of any potentially transgenic F_1 seedling progeny from the field collections was the use of sealed boxes to transport the doubly bagged sentinel plants and the bagged resident plant panicles during their transport to greenhouses.

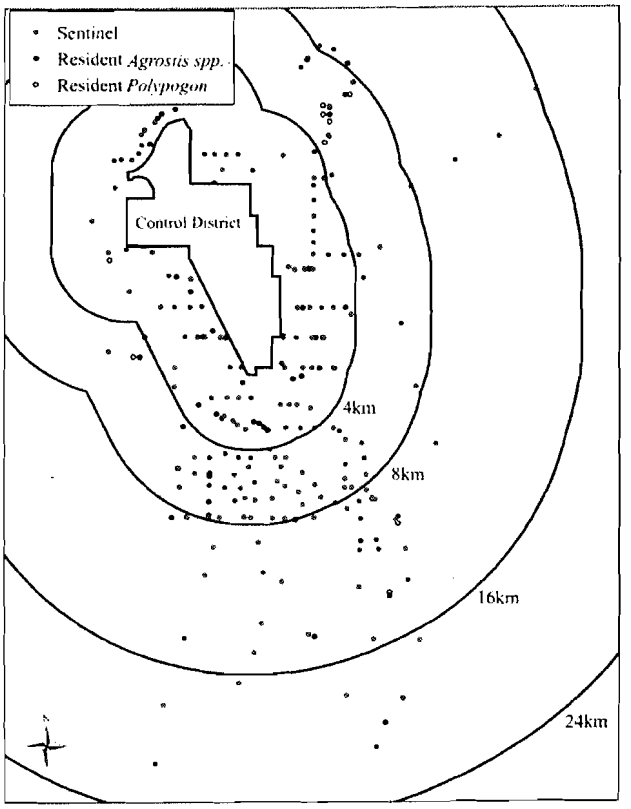

Fig. 1. Sampling design to determine gene flow from source fields within the control district to potentially compatible plants outside the control district. A total of 178 sentinel *A. stolonifera* plants (red circles) were placed outside the control district (6) near accessible public roads spaced 1.6 km apart in the north–south direction and 0.8 km apart in the east–west direction. Given a prevailing wind of 10 km/h from the north or northwest, 76 sentinel plants were located downwind from the control district in a 9.6-km-wide by 3.2-km-deep grid with ≈0.8-km spacing. Remaining sentinel plants were placed at 1.6-km intervals for the next 4.8 km and 3.2-km intervals for the next 6–10 km out to a distance of 16–21 km along six transects corresponding to major highways. In addition to the sentinel plants, 69 compatible resident *Agrostis* plants (black circles) of *A. stolonifera* and *A. gigantea*, plus 10 *P. monspeliensis* (open circles) located primarily along waterways and in moist soils, were included in the study.

Greenhouse Assays. Seeds harvested from sentinel and resident plants were chilled at 5°C for 7–10 days in moist sand and grown in trays of a peat-based potting medium (Seedling Mix no. 1, OBC Northwest, Canby, OR) in the greenhouse until the two-leaf stage and then sprayed with the field rate (2.3 liters/ha) of RoundUp herbicide by using a track sprayer (model RC-500-100-EP, Mandel, Guelph, ON, Canada). Seedlings that survived the initial spraying with the field rate of RoundUp or emerged after the spray event were subjected to spraying with herbicide at twice the field rate (4.6 liters/ha) ≈2 weeks later. Survivors of the second cycle of herbicide spraying identified as presumptive positives were confirmed by means of the TraitChek test.

Molecular Characterization. DNeasy Plant Mini kits (Qiagen, Valencia, CA) were used to extract genomic DNA from leaves of seedling progeny derived from 130 sentinel and 45 resident plants that were both herbicide resistant and TraitChek-positive for *CP4 EPSPS*. Primers for amplification and sequencing of a

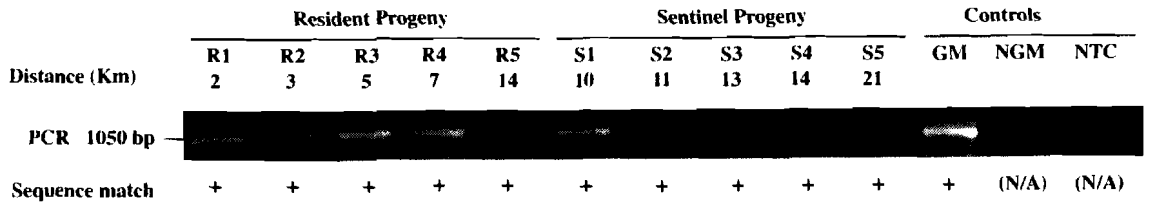

Fig. 2. Molecular confirmation of the presence of the engineered *CP4 EPSPS* herbicide-resistance gene. The presence of the *CP4 EPSPS* gene as verified in a subsample of TraitChek-positive progeny from resident (R1–R5) and sentinel (S1–S5) plants located at various distances from the control district perimeter. All PCR products had the same size and DNA sequence as that amplified from the GM-positive control (*A. stolonifera*, designated event ASR368). BLASTN searches (29) revealed that the DNA sequences also matched GenBank accessions AF464188.1, *Glycine max CP4 EPSPS* (score = 1,271, $E = 0.0$), and AY125353.1, a synthetic *CP4 EPSPS* construct. Negative controls included DNA from nonGM (NGM) *A. stolonifera*, variant Penncross, and a nontemplate control (NTC). +, positive sequence matches; N/A, not applicable.

1,050-bp segment of the *A. stolonifera CP4 EPSPS* coding region were designed with PrimerSelect (DNASTAR, Madison, WI) based on *Glycine max* (L.) Merr. *CP4 EPSPS* (GenBank accession no. AF464188.1). Amplifications with P217F (5′-ACTATGGGCCTCGTCGGGGTCTA-3′) and P218R (5′-GGCAGCCTTCGTATCGGAGAG-3′) were conducted for 40 cycles of 94°C for 30 sec, 64°C for 30 sec, and 72°C for 90 sec. PCR products were purified with QIAquick Gel Extraction kits (Qiagen). Cycle-sequencing reactions used BigDye v3.1 chemistry and the standard thermal profile suggested by the manufacturer (Applied Biosystems). Labeled fragments were purified with CleanSeq kits (AgenCourt Bioscience, Beverly, MA). Sequence data were collected on a Prism 3100 Genetic Analyzer (Applied Biosystems). Sequences were then compared with matching GenBank accessions by using BLASTN searches (29).

Statistical Analyses. The percentage of positive seedling progeny was calculated as the number of seedlings that survived two sprays with RoundUp and had positive TraitChek tests for the *CP4 EPSPS* gene divided by the total estimated number of seedlings germinated in the greenhouse. Maximum likelihood estimation was used to fit the two-parameter gamma distribution (30), $f(x) = x^{(\alpha-1)}\exp(-x/\beta)/(\Gamma(\alpha)\beta^{\alpha})$, where α and β are the model parameters and $\Gamma(\cdot)$ is a complete gamma function, to the observed distances from the control district perimeter at which positive seedling progeny were found. The adequacy of the gamma distribution was tested by using the one-sample Kolmogorov–Smirnov test (31, 32). The two-sample Kolmogorov–Smirnov test (33) was used to compare the probability distributions of the positive seedling progeny of sentinel and resident plants. Nonparametric kernel smoothing (34) was applied to percent positive seedling progeny to generate spatial maps of gene flow transfer for sentinel and resident plants separately. The estimation and hypothesis testing of the gamma distributions were performed by using S-PLUS v6.01 (Mathsoft, Cambridge, MA). Kernel smoothing and spatial maps were undertaken by using ARCMAP v8.3 and the ARCGIS SPATIAL ANALYST 8.3 (Environmental Systems Research Institute, Redlands, CA).

Results

Gene Flow to Sentinel and Resident Agrostis. Molecular analyses by PCR (see Fig. 2) and by sequencing (sequence data not shown) confirmed the presence of the *CP4 EPSPS* marker in seedling progeny that had survived two cycles of spraying with RoundUp herbicide. The sequence matched that of GenBank accession AF464188.1 for a *CP4 EPSPS* construct in glyphosate-resistant soybean (*G. max*). The highest relative frequencies of pollen-mediated gene flow to *A. stolonifera* sentinel and *A. stolonifera* and *A. gigantea* resident plants were observed within 2 km of the control district perimeter. Maximal distances at which gene flow was observed in sentinel and resident *A. stolonifera* and resident *A. gigantea* plants were ≈21 km, 8 km, and 14 km, respectively. Viable pollen dissemination distances for sentinel plants may be biased low because this distance of 21 km represented the limit of the sampling design (Fig. 1). An additional source of bias is that distances from source fields to the control district perimeter were unknown. Based on the one-sample Kolmogorov–Smirnov goodness-of-fit test, the empirical distribution of minimum distances of the 75 positive sentinel *A. stolonifera* plant locations (Fig. 3*A*) was adequately described by a gamma distribution with $\alpha = 0.93$ and $\beta = 4.1$ ($P = 0.67$); that of 16 positive resident *A. stolonifera* plant locations (Fig. 3*B*) was adequately described by a gamma distribution with $\alpha = 0.74$ and $\beta = 4.0$ ($P = 0.77$); and that of 13 positive resident *A. gigantea* plant locations (Fig. 3*C*) was adequately described by a gamma distribution with $\alpha = 0.74$

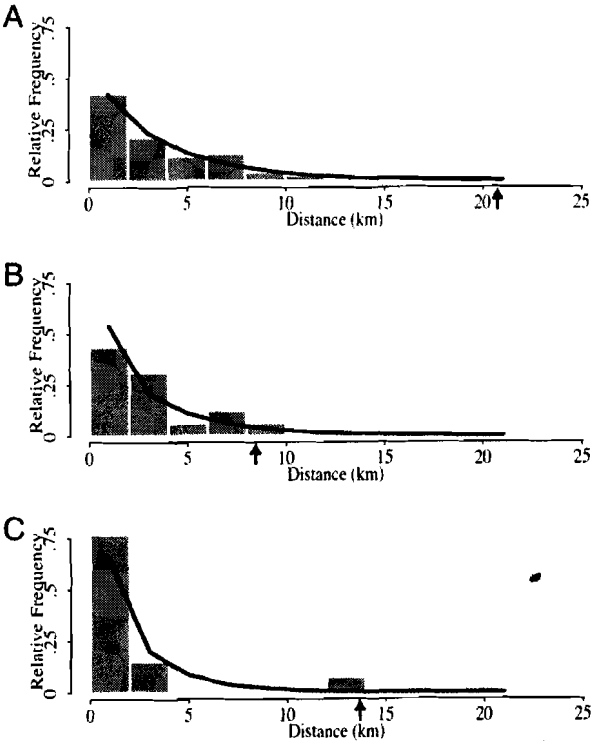

Fig. 3. Skewed distribution of GM bentgrass pollen-mediated gene flow to sentinel and resident plants in 2003. Based on the presence and expression of the *CP4 EPSPS* gene for herbicide resistance, relative frequencies of gene flow among sentinel and resident plant seedling progeny were highest within the first 2 km from the perimeter of the control district and decreased with distance. Arrows depict maximal gene flow distances that were observed. *A*, *B*, and *C* represent locations of sentinel *A. stolonifera*, resident *A. stolonifera*, and resident *A. gigantea* plants, respectively.

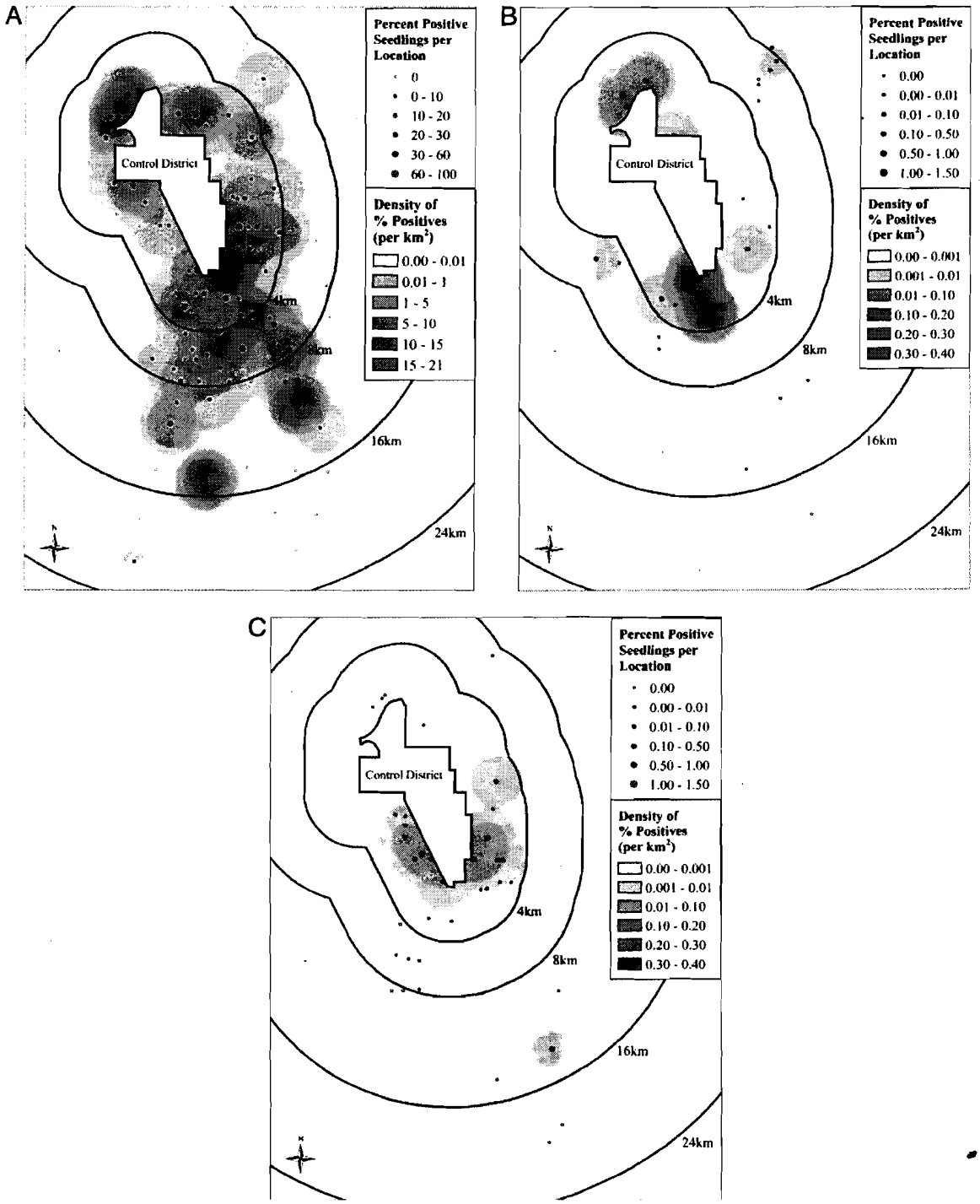

Fig. 4. Prevalence of gene flow based on percent positive seedling progeny of sentinel and resident plants at various distances from the control district perimeter. Kernel smoothing (34) was applied to percent positive seedling progeny (filled circles) of sentinel *A. stolonifera* plants (*A*), resident *A. stolonifera* plants (*B*), and resident *A. gigantea* plants (*C*) to generate spatial maps of the density of percentage positives. Open circles indicate locations where no positive seedling progeny were found. The highest densities of percent positive seedling progeny of sentinel and resident *A. stolonifera* plants occurred southeast and/or due south of the perimeter of the control district, in the direction of the prevailing winds.

and $\beta = 2.8$ ($P = 0.42$). The mean ($\alpha\beta$) and variance ($\alpha\beta^2$) of a gamma distribution decrease monotonically with respect to α and β. Consequently, higher α and β values indicate density distributions of viable pollen that hybridized with sentinel or resident plants that were spread farther from source fields. The gamma distributions for sentinel and resident *A. stolonifera* locations were not significantly different at the 0.05 level based on the two-sample Kolmogorov–Smirnov test ($P = 0.63$) but were significantly different from that for resident *A. gigantea* locations ($P = 0.031$ and 0.047, respectively). The mean distance

Table 1. Prevalence and incidence of *CP4 EPSPS*-positive plants and seedling progeny

Species	Plants with positive seedling progeny,* %	No. tested†	No. positive seedling progeny (%)
Sentinel	54	32,000	625 (2.00)
A. stolonifera	(75/138)		
Resident	53	565,000	157 (0.03)
A. stolonifera	(16/30)		
Resident	33	397,000	159 (0.04)
A. gigantea	(13/39)		
Resident	0	190,000	0 (0.00)
P. monspeliensis	(0/10)		

*Values in parentheses represent the ratio of plants with positive seedling progeny to the total number of plants.
†Number of seedling progeny tested in greenhouse.

from the perimeter of the control district ranged from 2.1 km for resident *A. gigantea* plant locations to 3.8 km for sentinel plant locations.

Spatial Patterns of Gene Flow. For both sentinel and resident *A. stolonifera* plants, the greatest spatial density of percent positive seedlings was found southeast and south of the control district in the direction of prevailing winds (Fig. 4 *A* and *B*). Positive seedlings derived from resident *A. gigantea* were found primarily east and west of the southern portion of the control district (Fig. 4*C*). In addition, some *CP4 EPSPS*-positive *A. stolonifera* seedling progeny were obtained from seeds harvested from plants near and below the northwest section of the control district perimeter (Fig. 4 *A* and *B*). This finding may be due to localized temperature gradients and wind conditions near the rim of the Deschutes River canyon, which brought viable pollen down to the canyon floor. In comparison, the percentage of positive sentinel plants was about an order of magnitude higher than that for resident plants.

Resident *Agrostis* typically were found in moist soils, e.g., riparian areas and along irrigation or drainage ditches. Most of the *A. stolonifera* resident plants with positive seedling progeny were located in sagebrush steppe or other nonagronomic land use areas (50% and 25%, respectively), whereas the majority (78%) of positive *A. gigantea* plants were located in agronomic production areas. Forty of 178 sentinel plants were lost to various causes, e.g., transplant shock and grazing. As shown in Table 1, hundreds of *CP4 EPSPS*-positive seedling progeny were found among *A. stolonifera* sentinel and resident plants and *A. gigantea* residents.

Discussion

Our multiple lines of evidence from greenhouse and laboratory tests document movement of viable GM creeping bentgrass pollen on a landscape level that encompassed ≈310 km². The gene flow evidence presented here contrasts quantitatively with previous studies with *A. stolonifera* (18, 25, 26) with significantly higher numbers of occurrences and maximally observed linear distances. The higher number of observed occurrences may reflect greater total acreage of source fields in this study (162 ha) as compared with much smaller experimental field plots of previously reported studies with *Agrostis* in which only several hundreds of plants served as pollen donors. The long period of flowering (estimated at 4–5 weeks rather than a more typical flowering period of 2–3 weeks for creeping bentgrass in the Willamette Valley), may have been due to asynchronous flowering of GM crop source fields. Potential causes of floral asynchrony include differences in cultivars, soil characteristics, and microclimates among source fields. The long gene flow distances we observed may, in part, reflect our sampling design, which purposefully looked at a range of distances in directions guided by historic information on prevailing winds (www.usbr.gov/pn/agrimet) as well as a 3-h window of assumed pollen viability (28). Our landscape level sampling design was distinct from "wagon-wheel" designs typically used for gene flow determinations in agronomic settings; i.e., with regard to its geographic scale of several hundreds of kilometers-squared rather than linear meters, in the broad variety of nonagronomic as well as agronomic habitats that it encompassed, and in the use of both sentinel and resident plants.

Lower frequencies of gene flow observed in resident *Agrostis* as compared with sentinel plants are likely primarily due to initiation of flowering of resident plants 2–3 weeks later than crop source fields. Pollen competition, i.e., pollen loads in the vicinity of patches of resident plants were higher than around individual sentinel plants, may also have reduced the relative availability of stigma sites and GM pollen in resident plants. Diverse factors (35) may have resulted in our lack of observations of gene flow to *P. monspeliensis* resident plants; two reasons we consider most likely are flowering of *P. monspeliensis* residents 2–3 weeks later than the bentgrass fields and their generally upwind locations.

Our results clearly document pollen movement and gene flow from large source populations of GM creeping bentgrass into much smaller numbers of resident *Agrostis* plants and individual sentinel plants of *A. stolonifera*. Conceivably, gene flow to resident plants from small-scale field trials of GM creeping bentgrass initiated within the control district before 2003 (www.agcomm.ads.orst.edu/agcomwebfile/edmat/html/sr/sr1046.9htm; ref. 36), e.g., by wind-dispersed pollen or seeds, may have contributed to the observations we report here. However, all tests done to date on leaf and panicle tissue samples of resident plants that produced *CP4 EPSPS*-positive seedling progeny in our greenhouse assays have proven negative for the marker. Efforts will continue over the next few years to identify potential establishment and recruitment of resident *Agrostis* that express the *CP4 EPSPS* marker. More detailed molecular analyses of positive seedlings and of maternal or paternal crop or resident plant parents are planned to distinguish hybridization events between GM crop and resident plants from GM crop seed dispersal. Multiyear sampling to monitor potential introgression of the *CP4 EPSPS* marker into resident populations and for potential effects on plant community structure and the ecological fitness of progeny also is planned.

In competitor–stress tolerator–ruderal characterization of plant functional types (13, 37), *A. stolonifera* is considered to have both competitive and ruderal features; thus, its invasive root and stolon growth can contribute to weediness, and new plants can be established either by seeds or by dispersal of stolon pieces (13, 15, 38, 39). The particular engineered trait for herbicide resistance (*CP4 EPSPS*) that we used as a selectable marker would not be anticipated *per se* to confer a selective advantage in the absence of herbicide selective pressure. However, in areas where weed control or restoration efforts are being practiced, hybrid *Agrostis* progeny resistant to glyphosate herbicide might be expected to have a selective advantage. Further studies should continue over the next few years within resident plant populations to monitor for introgression, spread, or extinction of the engineered *CP4 EPSPS* gene, and for potential effects on ecological fitness of progeny and plant community structure in various, largely nonagronomic habitats.

Biological confinement strategies (e.g., male sterility, gene insertion into organelles or into targeted chromosomes or chromosome sites) are of interest to try to restrict gene flow; however, recent reports (40, 41) suggest that gene leakiness may make fully effective, long-term containment of transgenes unlikely. Studies, such as the one reported here, that use both

sentinel- and resident-compatible plants in an appropriately large sampling design that includes nonagronomic and agronomic habitats may be useful to quantify potential rates of gene exchange between GM or conventional crops and nonagricultural resident plants when conducting assessments of ecological risks (35) and evaluating potential mitigation technologies (41). Similar approaches could be used to develop sampling designs to test for potential long-distance wind dispersal of GM seeds. Our methods and findings contribute significantly to the ongoing discussion about potential risks of gene flow from GM crops and thus are anticipated to be of interest to plant scientists, evolutionary biologists, ecologists, policy makers, and regulators.

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